Analysis of Gene Expression in Female American Lobsters (Homarus americanus) to Determine Reproductive Status by Oligonucleotide Microarray Analysis

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University of Prince Edward Island

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Charlottetown, P.E.I.

July, 2013

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Abstract

The American lobster (Homarus americanus) is harvested along the eastern seaboard of North America and forms the economic backbone of many small fishing communities in Atlantic Canada and the northeastern United States. The industry sets minimum size (carapace length) for harvesting American lobsters partially based on determining when females are sexually mature. Given the internal development of ovarian follicles (eggs) protected by a rigid exoskeleton, external assessment of sexual maturity is near impossible. Ovaries can be classified into 7 distinct developmental stages ranging from immature to mature based on a combination of factors including ovary colour, oocyte size, ovary factor, and time of year. Stage 4 ovaries are subdivided into stage 4a (developing) and 4b (mature) ovaries. Ovary stage 4b is important as it represents reproductive commitment. This means that once ovaries progress to stage 4b, they can no longer delay maturation and must continue through until extrusion of eggs onto the pleopods. To stage ovaries and accurately estimate the size at the onset of maturity, female American lobsters must be sacrificed to examine the ovary both grossly and histologically. The discovery of a non-lethal biomarker would result in more accurate ovary staging while allowing the return of the female to the spawning population. Several studies have examined specific reproductive endocrine processes in the American lobster, including the roles of vitellogenin, crustacean hyperglycemic hormone, and gonad-inhibiting hormone. However, no molecular approaches have been attempted to evaluate reproductive status in female American lobsters.

This study was the first to examine genome-wide expression changes of reproductive female American lobsters using a novel 14,592 feature, spotted oligonucleotide

microarray. The genetic information on the microarray was ~40% functionally annotated based on comparisons with publically available molecular databases. A reference design was used to allow stage specific comparisons between individual ovary stages. Four tissues (ovary, hepatopancreas, eyestalk, and haemocyte pellet) were pooled prior to cDNA synthesis to reduce reagents used and to capture gene expression data from these reproductively important tissues. Therefore, all results were total gene expression in the pooled samples. This study determined 1,774 genes to be statistically significant based on a one-way ANOVA, with 569 functionally annotated genes. The latter were involved in a variety of important biological processes including reproduction, development and growth. A total of 12 genes of interest were chosen for validation using reverse transcription quantitative polymerase chain reaction (RT-qPCR): four vitellogenin genes, two ovary development related proteins, egg-derived tyrosine phosphatase, estradiol-17-β-dehydrogenase 12-B-like, 97 kDa heat shock protein, quaking protein A, growth-hormone inducible transmembrane protein, and inhibitor of growth protein 1-like.

Four genes represented distinct copies of vitellogenin, which is converted into vitellin, an important ovary/egg related protein involved in egg yolk. One of these genes was the complete Homarus americanus vitellogenin (HaVg1). A second gene, HaVg2, was 85% similar at a nucleotide level and 74% similar at a protein level with HaVg1. The third EST, given the name HaVg3, was 55% similar at nucleotide and protein levels with HaVg1. The final vitellogenin EST, named HaVg4, was 65% similar at a nucleotide level and 44% similar at a protein level with HaVg1. Three of these vitellogenins (HaVg2, HaVg3, and HaVg4) were unique to this study. The expression profiles of all 4 vitellogenins showed progression from downregulation at stage 2 to upregulation by

stage 5, where stages 4a, 4b, and 5 are upregulated. This pattern was consistent between microarrays and RT-qPCR and agreed with previous literature in crustaceans.

With the increased anthropogenic and environmental pressures on American lobster stocks, including harvesting and global climate change, this study has only begun to examine potential genetic variations in the American lobster. Future research should examine gene expression on an individual tissue level as genes are regulated on a tissue level. Only 32% of our statistically significant genes were functionally annotated. Further functional annotation may highlight genes associated with reproduction and commitment.

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Dedication

This thesis has had it's trials and without the support and love of the following individuals, I may not have finished. I am greatly endebted to these individuals and words cannot express how greatful I truely am.

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List of Abbreviations

µg	Microgram
µL	Microlitres
µM	Micromolar
1G4F	1% Glutaraldehyde:4% Formaldehyde
ANOVA	Analysis of Variance
aRNA	Amplified RNA
AVC	Atlantic Veterinary College
AVCLSC	Atlantic Veterinary College Lobster Science Centre
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CHH	Crustacean Hyperglycemic Hormone
CNRQ	Calibrated Normalized Relative Quantities
CoA	Coenzyme A
Ct	Cycle Threshold
DDT	Dithiothreitol
DEPC	Diethylpyrocarbonate
DFO	Department of Fisheries and Oceans
diH2O	Deionized Water
DL	Daylength
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
E-17-β-D	Estradiol-17-β-dehydrogenase 12-B-like
EDTA	Ethylenediaminetetracetic Acid
EDTP	Egg-Derived Tyrosine Phosphatase
Egh	Egghead
EIA	Enzyme Immunoassay
EST	Expressed Sequence Tag
FA	Farnesoic Acid
FDR	False Discovery Rate
FOM	Figure of Merit
FRCC	Fisheries Resource Conservation Council
g	Gravity
GAL	GenePix Array List
GFP	Green Fluorescence Protein
GHITM	Growth-Hormone Inducible Transmembrane Protein
GIH	Gonad Inhibiting Hormone
GOI	Gene(s) Of Interest
GSH	Gonad Stimulating Hormone
HaVg1	Homarus americanus Vitellogenin 1
HaVg2	Homarus americanus Vitellogenin 2
HaVg3	Homarus americanus Vitellogenin 3
HaVg4	Homarus americanus Vitellogenin 4
HCl	Hydrogen Chloride
HIST	Histology
HSP	Heat Shock Protein
IDT	Integrated DNA Technologies
ING1	Inhibitor of Growth Protein 1
IVT	In vitro Transcription
kDa	Kilodalton

LG	Lamina ganglionaris
LFA	Lobster Fishing Area
LOWESS	Locally Weighted Scatterplot Smoothing
M	Molar
MA	Microarray
MAGE	Microarray Gene Expression
MAGE-ML	Microarray Gene Expression – Markup Language
MAGE-OM	Microarray Gene Expression – Object Model
ME	Medulla Externa
MeV	Multiexperiment Viewer
MF	Methyl Farnesoate
MI	Medulla Interna
MIAME	Minimum Information About A Microarray Experiment
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MIH	Moult Inhibiting Hormone
MO	Mandibular Organ
mRNA	Messenger Ribonucleic Acid
MT	Medulla Terminalis
NaOH	Sodium Hydroxide
No-RT	No Reverse Transcription
NTC	No Template Control
OC	Ovary Colour
ODRP	Ovary Development-Related Protein
O_f	Ovary Factor
ON	Optic Nerve
OS	Oocyte Size
PCR	Polymerase Chain Reaction
PEI	Prince Edward Island
pmol	Picomole
PMT	Photomultiplier Tube
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SE	Standard Error
SG	Sinus Gland
SNR	Signal to Noise Ratio
SOM_{50}	Size where 50 percent of females are sexually mature
SSC	Saline-Sodium Citrate
TG	Thoracic Ganglion
Tm	Melting Temperature
USA	United States of America
Vg	Vitellogenin
XML	Extensible Markup Language
XO	X-Organ
YO	Y-Organ

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CHAPTER 1: GENERAL INTRODUCTION

1.1 General Overview of American Lobster Industry

The American lobster, Homarus americanus (Milne-Edwards) was considered, up until 1850, a poor man's food being fed to servants and prisoners alike. It was big cities like New York, Boston, and Chicago where the demand for American lobster as a premium restaurant dish initially increased. This was the true beginning of the mental switch to, what is now, one of the most highly demanded commercial aquatic food species. The fishery has become the most lucrative annual seafood export in Canada, representing nearly $620 million in 2011 (Department of Fisheries and Oceans 2013). American lobster's natural habitat is along the Atlantic Ocean from North Carolina, USA to the Southern part of the Labrador Sea, and the livelihood of many coastal communities in Maine, Nova Scotia, New Brunswick, Prince Edward Island, and Quebec rely heavily on the American lobster fishery. There are over 10,000 lobster fishing licenses and the industry employed more than 25,000 people in 2009.

American lobster harvesters in Canada can only legally use baited wooden or wire traps to attract and capture lobsters. They are designed and equipped with an escape vent to minimize the capture of undersize American lobsters that are illegal to possess and have to be returned to the water. Traps sit on the ocean floor attached to buoys at the surface and are usually hauled on a daily basis in the Gulf of St. Lawrence. Legal sized American lobsters have their claws banded and are placed in crates or holding tanks with circulating water on board the boat. Before being re-set, traps are re-baited, and placed back in the water (Miller 1995; Miller 2003; Department of Fisheries and Oceans 2009).

In the Canadian Maritimes, there are 45 Lobster Fishing Areas (LFA) including one offshore and one closed for conservation. These LFAs were not established based on the biology of the American lobster, but rather on historical and political considerations. Each LFA has specific regulations on effort (i.e. the number of traps allowed, the number of licences, the length and timing of the lobster fishing season) and the type of American lobster that can be legally caught (i.e. minimal legal size, berried females are prohibited). Without limitations on how many pounds of lobster can be caught, especially in the past few years where harvesters are making less per pound, there is an increased possibility of overfishing to make up for lost wages (Miller 2003; Department of Fisheries and Oceans 2011).

1.2 Fisheries Resource Conservation Council

The Fisheries Resource Conservation Council (FRCC) was a non-governmental council composed of representatives from industry and universities that reviewed fisheries at the request of the Minister of the Department of Fisheries and Oceans Canada (DFO) and made recommendations to improve the sustainability of various fisheries. The American lobster fishery was reviewed in 1995 and again in 2007. Recommendations were similar in both years. In 2007, the FRCC found that little from the 1995 review had been instituted to reduce fishing effort and maintain a healthy American lobster resource. Both reviews had significant concerns that exploitation rates were increasingly high. They also feared that most of the American lobsters are harvested before they reach sexual maturity (FRCC 2007).

During the 2007 review, the council put particular emphasis on increasing the minimum carapace size to the size at onset of 50% maturity (SOM_{50}; size at which 50% of females

are mature). At the time of the review, Southwest Nova Scotia had a minimum legal carapace size of 82.5 mm and the SOM_{50} was 97 mm. This would mean that the majority of the females would be harvested before they release one brood of eggs (FRCC 2007). In 1996, LFA 26a had a minimum carapace size of 65.1 mm and the SOM_{50} was 72.0 mm; most American lobsters were being harvested prior to maturation (Comeau and Savoie 2002). It is estimated that for every 10,000 American lobster larvae released, only 1 to 10 will make it to maturity (Department of Fisheries and Oceans 2009).

1.3.1 General American Lobster Biology

American lobsters are decapod crustaceans, literally meaning "ten-footed". For the American lobster, this consists of four pairs of walking legs, or periopods, and two claws, the crusher and pincer claws. American lobsters have a hard calcified exoskeleton, which is normally greenish in colour, and to grow must shed this exoskeleton, a process known as moulting. While their shell is still soft, American lobsters take in water and can grow up to 15% in size and 40-50% in weight. A quick method for distinguishing pre-moult from non-pre-moult American lobsters is to clip the tip of a pleopod (swimmeret) to examine the extent the new shell forming has pulled away from the old shell (Aiken 1973; Department of Fisheries and Oceans 2009). The most accurate method for determining moult stage is by histological examination (Drach 1939). American lobsters are pelagic during their larval phase (stages I-IV) moulting three times before reaching the transitional stage IV (post-larvae). At stage IV, they establishing a benthic lifestyle with cryptic behavior, hiding within cobble and feeding opportunistically until they are large enough to be protected by their carapace when they will begin emerging to feed and search for larger hiding places (Department of Fisheries and Oceans 2009).

1.3.2 Reproduction of Female American Lobsters

Female American lobsters mature their eggs internally. The ovaries lie dorsally in the lobster's body cavity covered by their hard exoskeleton, making it nearly impossible to determine the sexual stage of the ovaries through external observation (MacDiarmid and Sainte-Marie 2006). American lobsters will begin to reproduce ~6 to 8 years after hatching, allowing the lobster to put more energy into somatic growth as opposed to gonadal growth. Once large enough to carry a brood of eggs, the female will moult and be inseminated by 1 or more males (Jones et al. 2003; Gosselin et al. 2005; MacDiarmid and Sainte-Marie 2006). Reproduction in females is also dependent on many environmental factors, including daylength, temperature, food availability, and degree-days (Talbot and Helluy 1995; Waddy et al. 1995). Sperm can be carried internally in specialised organs for up to three years before it is used (Waddy and Aiken 1986; MacDiarmid and Sainte-Marie 2006).

Females generally follow a two-year reproductive cycle; ~20 % of females can actually complete the cycle in one year. Females following a two-year cycle will moult and mate within the same summer, overwintering with eggs developing internally and then extruding the eggs the next summer, fertilizing them in the process. The eggs are then held externally on the pleopods until the following year when they will be released as larvae. Females with a one year cycle will moult and mate early in the summer, extrude and fertilize eggs the same summer, and release larvae the next summer. The female reproductive cycle is influenced by environmental factors, mainly temperatures (Waddy and Aiken 1992; Comeau and Savoie 2002). Currently, there is no external way of classifying American lobsters into 1 of the seven ovary development stages. The internal

factors for staging ovaries have also been widely disputed (Waddy and Aiken 2005). For these internal measures, the lobster must be sacrificed for examination of internal characteristics such as ovary colour, oocyte size, and ovary factor (O_f) (Table 1.1). Ovaries vary in colour from white to a dark green. Oocyte size is also difficult to determine. Oocytes can vary in size within one female, since not all oocytes mature at the same rate. This means that there must be a range in oocyte size for determining the ovary stage. The ovary factor is a function which takes into consideration the total wet ovary weight and the carapace length. The equation follows:

$$O_f = 10 \times \frac{ovary\ wet\ weight(mg)}{carapace\ length\ (cm)^3}$$

This equation, combinated with ovary colour and oocyte size, allows for a more comprehensive estimation of the ovary status (Waddy and Aiken 2005).

Table 1.1: Criteria for determining ovary stages in the American lobster (Homarus americanus). Female lobsters with mature ovaries are committed to proceed through with oocyte extrusion. They will not moult until they spawn and broods are hatched (Adapted from Waddy and Aiken 2005).

Immature Ovary	**Developing Ovary**	**Mature Ovary**	**Spent/Resorbing Ovary**
Stage 1:	**Stage 2**:	**Stage 4b** (Spring):	**Stage 7**:
Ovary - white	Ovary - yellow, orange,	Ovary - medium to dark	Ovary - large, limp,
Oocytes <0.5 mm	beige, or pale green	green	white or yellow,
O_f <100	Oocytes <0.8 mm	Oocytes 0.8-1.6 mm	may have residual
	O_f <100	O_f 200-325	green oocytes
	Stage 3:	**Stage 5**:	
	Ovary - light to medium	Ovary - dark green	
	green	Oocytes 1.0-1.6 mm	
	Oocytes <1.0 mm	O_f >325	
	O_f <200		
		Stage 6:	
	Stage 4a (Autumn):	Ovary - dark green	
	Ovary - medium to dark	Oocytes 1.4-1.6 mm	
	green	O_f >400	
	Oocytes 0.1-1.6 mm		
	O_f <200	**Stage 6a**:	
		Post-ovulation (oocytes	
		free in ovary)	

The integral stage of ovary maturity falls between 4a and 4b. After an ovary reaches stage 4b they are committed to mature and will continue until eggs are extruded or resorbed. Currently, the length of time that the ovaries can be held at stage 4a is unknown, as lobsters must be sacrificed to determine the true ovary stage. The determination of American lobsters with stage 4b ovaries is crucial as it aids in determining maturity, which is used for SOM_{50}. Another useful measure in the latter stages of ovary development is the visual presence of cement glands on the pleopods. Cement glands

secrete a substance to aid in attachment of the fertilized eggs to the abdomen (Aiken and Waddy 1982; Comeau and Savoie 2002).

1.4 SOM_{50}

The SOM_{50} is one of the criteria currently used in setting the minimum legal carapace length in each LFA. Internal features of the ovaries need to be examined to establish the SOM_{50} for the 40+ LFAs in Canada. Hence, DFO needs to sacrifice thousands of American lobsters in order to ensure accuracy of their results. DFO uses a dichotomous key for consistency of determining maturity in the female lobsters (Figure 1.1).

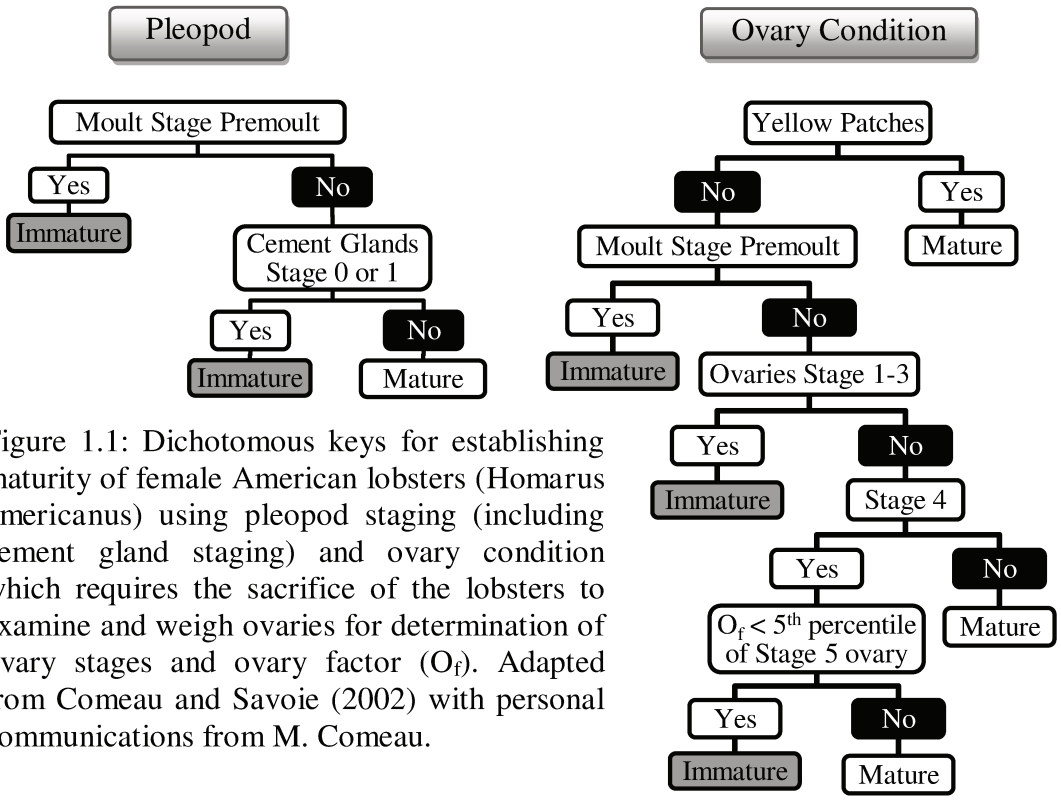

Figure 1.1: Dichotomous keys for establishing maturity of female American lobsters (Homarus americanus) using pleopod staging (including cement gland staging) and ovary condition which requires the sacrifice of the lobsters to examine and weigh ovaries for determination of ovary stages and ovary factor (O_f). Adapted from Comeau and Savoie (2002) with personal communications from M. Comeau.

This key, however, is subject to widespread debate over the different staging methods, especially as it relates to reproductive commitment: ovary stages 4a and 4b (Comeau and Savoie 2002; Waddy and Aiken 2005). Waddy and Aiken (2005), in response to an article published by Comeau and Savoie (2002) on the maturity status of female

American lobsters in the Gulf of St. Lawrence, stated that the methods of determining maturity was not in accordance with methods used for more than 20 years. They stated that the determination of maturity relies on the proper estimation of spawning or moulting. This is difficult; Comeau and Savoie (2002) stated that ~20% of American lobsters in the southern Gulf of St. Lawrence will follow a one-year maturation cycle where they moult and extrude eggs in the same year, instead of the typically accepted two-year cycle where this happens in alternate years. This confusion could be avoided if there were an objective test that could be used to properly differentiate those American lobsters in ovary stage 4a versus those in stage 4b.

1.5 Female Reproductive Endocrinology

Many studies have looked at the endocrinology of reproduction in crustaceans (Paulus and Laufer 1987; Chang et al. 1990; Fingerman 1997; de Kleijn et al. 1998; Chan et al. 2003; Eichner et al. 2008; Karoonuthaisiri et al. 2009; Brady et al. 2012). In reproduction, there are both inhibitory and stimulatory proteins and hormones which control the timing and rate of reproduction. The control of reproduction for decapod crustaceans is mainly achieved by the X-organ (XO), the Y-organ (YO), the mandibular organ (MO), and the thoracic ganglion (TG) (Nagaraju 2011). The XO is located within the eyestalk of crustaceans (Figure 1.2).

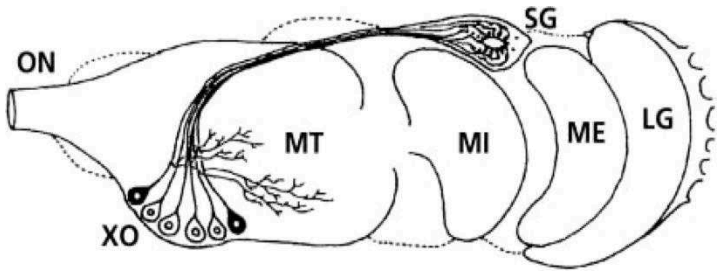

Figure 1.2: Crustacean eyestalk showing the location of the X-organ (XO), sinus gland (SG), lamina ganglionaris (LG), medulla externa (ME), medulla interna (MI), medulla terminalis (MT), and the optic nerves (ON) (Serrano et al. 2004).

The XO produces gonad inhibiting hormone (GIH) and crustacean hyperglycemic hormone (CHH) (de Kleijn et al. 1998; Fanjul-Moles 2006). GIH controls maturation in decapod crustaceans, in conjunction with gonad stimulating hormone (GSH) produced in the thoracic ganglion (Nagaraju et al. 2003; Nagaraju 2011). GIH in female crustaceans inhibits secondary vitellogenesis (Charniaux-Cotton and Payen 1988; de Kleijn et al. 1998), while other researchers have shown by using a heterologous bioassay that GIH is involved in the inhibition of the onset of vitellogenesis (Charniaux-Cotton 1985; Soyez et al. 1987; de Kleijn et al. 1998).

CHH can be separated into two immunoreactive groups (A and B). Both CHH groups have hyperglycaemic activity, regulating hemolymph glucose levels. CHH-B also stimulates oocyte growth whereas CHH-A has moult-inhibiting activities (Tensen et al. 1989; Chang et al. 1990; de Kleijn et al. 1998).

De Kleijn et al. (1998) have also mapped the relationship among GIH, CHH-A, and CHH-B messenger RNA (mRNA) levels in American lobster hemolymph during the biannual reproductive cycle (Figure 1.3). GIH is expressed in immature and previtellogenic animals. This demonstrates the inhibitory function of GIH due to the decrease in levels at late previtellogenesis, vitellogenesis, and mature stages (de Kleijn et al. 1998). Vitellogenesis in the American lobster begins in early March and can continue through to September (Tiu et al. 2009). This is consistent with research by Waddy and Aiken (2005) where vitellogenesis only occurred when water temperatures were above 5°C.

Due to the proposed GIH and CHH complexes moult-inhibiting roles, the lull in mRNA expression of these two complexes in the previtellogenic stage (Figure 1.3) may allow

moulting to occur. This would allow the female to mate with a male while in a soft-shell state making it possible for the male to insert the sperm sack (de Kleijn et al. 1998).

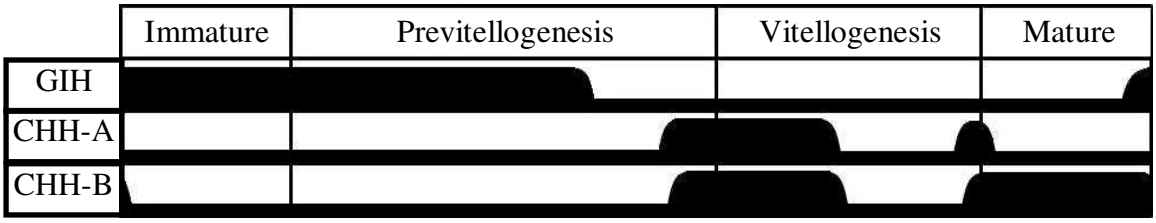

Figure 1.3: Synchronization of hemolymph levels of gonad inhibiting hormone (GIH) and crustacean hyperglycaemic hormones A and B (CHH-A and CHH-B respectively) over the course of the female American lobster's (Homarus americanus) reproductive cycle. Modified from de Kleijn et al. 1998.

Another crucial protein in the development of ovaries is vitellogenin (Vg). Vg is the precursor to the egg yolk protein, vitellin, and, in a variety of crustacean species (Cherax quadricarinatus, Penaeus monodon, Homarus americanus), is synthesized in the ovary (intraovarian) and/or hepatopancreas (extraovarian) depending on the stage of vitellogenesis (Tiu et al. 2008; Ferre et al. 2012). Vitellogenin produced in the hepatopancreas is secreted into the hemolymph and is transported to the developing oocyte within the ovary. The free vitellogenin then binds to the vitellogenin receptor, is actively transported into the oocyte via receptor-mediated endocytosis, and combines with polysaccharides and lipids to form yolk granules (Tiu et al. 2008; Tiu et al. 2009).

In ridgeback rock shrimp (Sicyonia ingentis), hemolymph vitellogenin levels increased steadily, peaking at stage 4 (Figure 1.4). There was, however, no differentiation between stage 4a and 4b ovaries. These levels are comparable to other crustaceans over the course of ovary development (Tsukimura 2001). There are, however, differing vitellogenin levels in several tissues of other crustaceans (Tsutsui et al. 2000; Phiriyangkul et al. 2007; Ding et al. 2010). For example, in the banana shrimp (Fenneropenaeus merguiensis), ovarian vitellogenin peaked in expression at ovary stage 2 and, in hepatopancreas,

steadily increased to ovarian stage 4 (Phiriyangkul et al. 2007). This differentiation could relate to an increased need for extra-ovarian vitellogenin just prior to reproductive commitment.

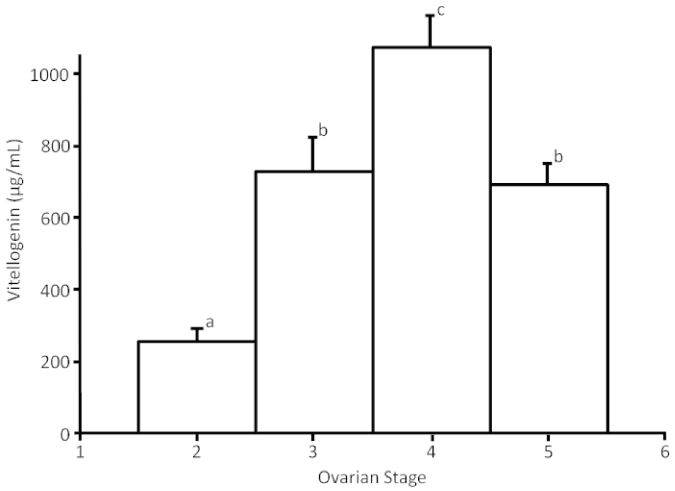

Figure 1.4: Hemolymph vitellogenin levels in the ridgeback rock shrimp (Sicyonia ingentis) over six ovary stages, peaking at stage 4. Stages with the same letter are not statistically different ($P < 0.05$) (Tsukimura 2001).

The chemical nature of gonad stimulating hormone (GSH) produced in the thoracic ganglion, has not been fully investigated. One study isolated GSH from the American lobster and injected it into whiteleg shrimp (Litopenaeus vannamei) which induced spawning. This suggests that this hormone is highly conserved across crustaceans or the presence of American lobster GSH induced the production of GSH in the shrimp (Subramoniam and Kirubagaran 2010).

Methyl farnesoate (MF) and farnesoic acid (FA) are produced in the mandibular organ of crustaceans. Methyl farnesoate is the precursor to insect juvenile hormone and could have gonadotrophic functions (Nagaraju et al. 2003; Subramoniam and Kirubagaran 2010; Tiu et al. 2010). However, there have been conflicting reports on the role of MF in reproduction. In the crayfish (Cherax quadricarinatus) and the red crab (Charybdis

feriatus), MF positively affects vitellogenesis (Soroka et al. 2000; Mak et al. 2005). In the American lobster, when female mandibular organs are removed, the animals continued to spawn normally. MF levels are high during the winter when vitellogenic activity is low and diminish to undetectable levels in the spring when vitellogenesis increases. MF alone may not control vitellogenin production in the same way as occurs in other crustaceans (Tsukimura and Borst 1992; Subramoniam 2000; Tiu et al. 2009; Subramoniam and Kirubagaran 2010; Tiu et al. 2010). However, FA, a precursor to MF, injected into hepatopancreas fragments resulted in a significant increase in extraovarian American lobster vitellogenin expression (Tsukimura and Borst 1992; Subramoniam and Kirubagaran 2010). FA in American lobsters may play the same role as MF does in other crustaceans. There may be some significant differences centered around FA over ovary stages.

Dopamine and serotonin are neurotransmitters which are important in gonadal development of decapod crustaceans (Sarojini et al. 1995; Tinikul et al. 2008). The role of serotonin, or 5-hydroxytryptamine, is suggested to have an inhibitory effect on the release of GIH from the eyestalk thus inducing gonad development in sand fiddler crabs, Uca pugilator, red swamp crayfish, Procambarus clarkii, black tiger shrimp, Penaeus monodon (Sarojini et al. 1995; Fingerman 1997; Wongprasert et al. 2006; Tinikul et al. 2008). Dopamine has an inhibitory effect on gonad development in sand fiddler crabs, Uca pugilator, and red swamp crayfish, Procambarus clarkii (Fingerman 1997; Tinikul et al. 2008). In the giant freshwater prawn, Macrobrachium rosenbergii the level of serotonin was quantified and increased from ovary stage I until reaching its highest concentration at ovary stage IV. Dopamine, however, was at its highest concentration at

ovary stage I and decreased until reaching the lowest concentration at stage IV (Sarojini et al. 1995; Fingerman 1997; Tinikul et al. 2008). Apparently, serotonin and dopamine have an inverse relationship in the reproductive process.

Although these endocrine processes have been studied in many crustaceans, very few have examined the changes over ovary stages especially as it relates to the American lobster. Methods currently available can be quite costly and time consuming. They do not allow examination of the expression of the transcriptome. For example, one study of enzymes in the Kumura prawn (Marsupenaeus japonicas) required several enzyme immunoassay (EIA) kits (Okumura and Sakiyama 2004). This technology allowed the examination of specific genes but there may be genes which are differentially expressed in the hemolymph which are being overlooked. Microarray technology combined with RT-qPCR analysis could aid in discovering new genes involved in reproduction and evaluating changes in known genes that could be used for future monitoring of reproductive status.

1.6 Microarray Technology

Microarrays allow for the monitoring of changes in expression of thousands of genes in a single, high throughput experiment. Microarrays are useful for the discovery of new genes associated with a particular biological event and for monitoring gene expression at a particular point in time in one or several tissues (Nettleton 2012). Historically, arrays have been made using complementary DNA (cDNA) or oligonucleotides adhered to glass slides with the latter providing more DNA sequence information per slide (Morey et al. 2006). Microarray experimental design consists of, but is not limited to, reference design, loop design, direct comparison, and balanced block designs (Figure 1.5). There are both

indirect and direct designs for experiments and several studies have explored which is most suitable under varying conditions and outcomes (Kerr and Churchill 2001; Quackenbush 2005; Altman and Hua 2006; Slonim and Yanai 2009).

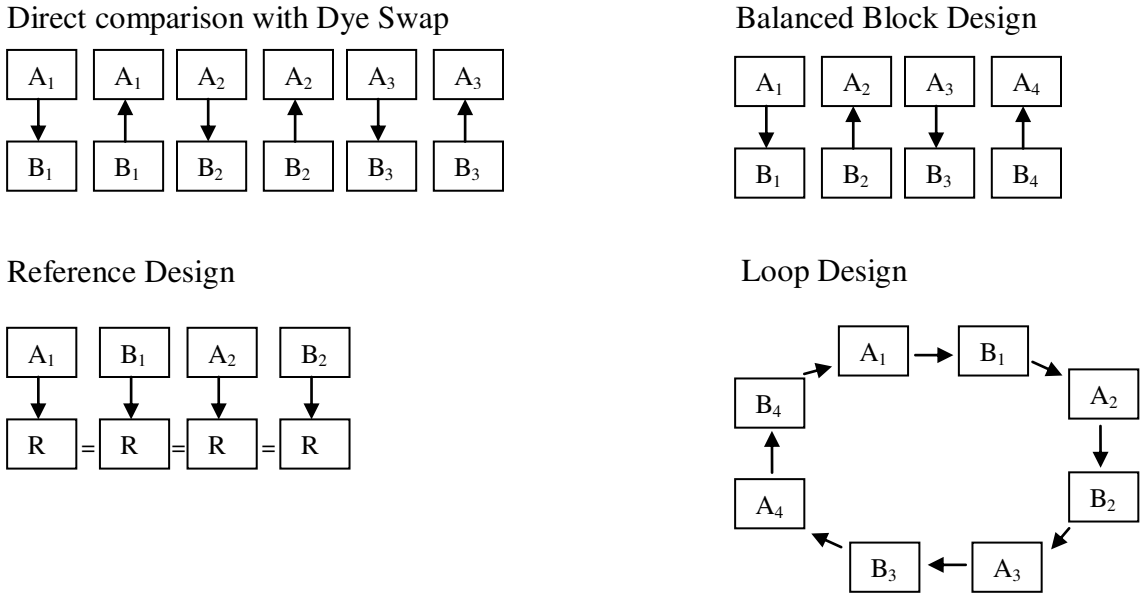

Figure 1.5: Four different experimental designs for microarray analysis. Boxes represent individual animals, A and B's represent different treatments, and subscripts represent biological replicates. Arrows represent dyes. The tail represents the opposite dye colour than the arrow head (Modified from Quackenbush 2005).

Direct comparisons are the simplest design and easiest to interpret. They are generally used when testing two distinct samples, e.g. treated versus non-treated (control). To decrease the concerns associated with dye biases, dye swapping has been used where the two differential fluorophores are swapped. For example, if A_1 was labelled with AlexaFluor 555 and B_1 with AlexaFluor 647 during the first microarray experiment, then the second microarray experiment would combine A_1 with AlexaFluor 647 and B_1 with AlexaFluor 555. The resulting difference in expression levels is then averaged between replicates to account for any bias associated with the binding affinity of the dyes. When a direct comparison is desired but there is limited quantity of sample available and dye

biases are a realistic concern, a balanced block design can be used. In contrast, reference designs compare the samples to a common RNA sample (reference). A common approach for developing a reference sample is to pool samples of RNA from each experimental sample. Some other forms include comparing to time 0 during a time course experiment, using a wild-type animal to compare to mutant animals, or to use a renewable "universal" RNA pool sample. A loop design is one of the most difficult to analyze because comparisons among samples begin to be much less precise as the number of samples involved increases (Ewens and Grant 2005; Quackenbush 2005; Altman and Hua 2006; Slonim and Yanai 2009; Nettleton 2012).

These designs can include both biological and technical replicates. Biological replicates use independent RNA samples from the same or different individual animals to repeat measurements made in an experiment. Technical replicates use the same RNA sample to test for variations in an experiment. Technical replicates can be divided into within-slide replication and between-slide replication. Within-slide replication tests the binding of sample to multiple probes representative of a single gene on one microarray. Between-slide replication tests the same hybridization conditions over multiple microarrays (Quackenbush 2005; Nettleton 2012).

Normalization is required for the analysis of data from microarrays. Normalization adjusts array data for effects caused by technical variations, not biological variations. Once the data is normalized, comparisons between datasets are possible. Normalization is a process which balances the intensity of channels to account for variations in labelling and hybridization efficiencies. Normalization can also adjust the brightness of the microarray based on background intensity (Smyth and Speed 2003; Quackenbush 2005;

Mecham et al. 2010). Without normalization, further analyses such as clustering or statistical analyses would not be accurate due to variation in raw data.

Microarray data can be clustered in several ways that allow detection of similarities between individual microarrays and between genes. The two most common clustering methods for microarray analysis are hierarchical clustering and K-means (Quackenbush 2005; Do and Choi 2008). Hierarchical clustering groups genes or microarrays that are the most similar together (Figure 1.6).

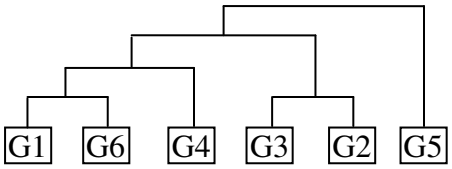

Figure 1.6: Hierarchical clustering of genes. Different numbers represent different genes. Genes (G1-G6) which are closest to one another are the most similar (Modified from Quackenbush 2005).

K-means or medians-clustering requires the user to specify the number of clusters desired. Genes are then randomly placed into a cluster and the mean or median expression profile for each cluster is calculated. The genes are then moved to the cluster with the closest mean or median expression profile according to the individual gene expression profile (Quackenbush 2005; Do and Choi 2008). The cluster's expression profiles are then calculated again and genes are shuffled. This continues until the genes can no longer be moved to another cluster or repeated until a user-specified number of moves are achieved (Figure 1.7).

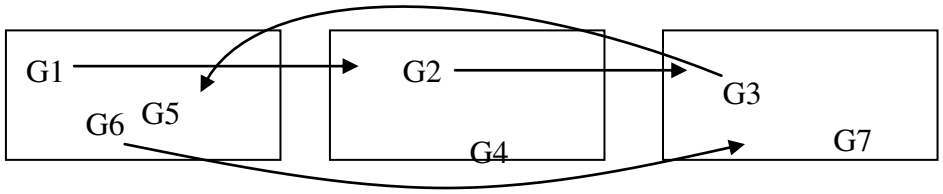

Figure 1.7: K-means clustering analysis. Boxes represent clusters, numbers represent individual genes (G1-G7), arrows represent the shuffle of genes to new clusters.

The guidelines known as "Minimal Information About a Microarray Experiment" (MIAME) must be followed to publish the results of microarray experiments (Brazma et al. 2001). The generally accepted data format is "MicroArray Gene Expression" (MAGE) (Spellman et al. 2002). Both of these standards allow for publishing uniformity. MIAME is defined in five separate parts: experimental design, samples, hybridizations, measurements, and microarray design (Brazma et al. 2001). Experimental design is explained by giving a brief description of the goal of the experiment with keywords, steps taken for quality control, and experimental factors. For each sample, the origin, any manipulation performed, and technical protocols must also be explained. If used, the author must also explain external (spike) controls. The protocol used for hybridization must also be included. The raw data, the normalized data and normalization method employed from the microarray experiment must also be made available. Finally, the microarray design, features, and reporter annotations must be included in each article. MAGE is used to upload the files required by MIAME to online databases such as the Gene Expression Omnibus (GEO) from NCBI ((http://www.ncbi.nlm.nih.gov/geo/). There are two types of MAGE – Object Model (OM) and MAGE – Markup Language (ML). MAGE-OM uses unified modelling language, which uses graphical representation depicting the relationships between different entities. MAGE-ML is derived from MAGE-OM and is based on Extensible Markup Language (XML) (Brazma et al. 2001; Quackenbush 2005).

All of these languages are used to create consistent microarray result reporting which increases confidence in results. These standards also simplify complex results into a

format which is easier to understand. Although most microarray experiments are not performed on crustaceans, the popularity in crustaceans is beginning to increase.

An experiment using this technology was done on Penaeus monodon to examine ovarian maturation (Brady et al. 2012). In this experiment, gene expression of the cephalothorax, which included hepatopancreas, Y-organ, and mandibular organ, and eyestalk as it related to ovary maturation of wild-caught and captive-reared individuals was examined. The cephalothorax and eyestalk were used because of their neuropeptide and hormone regulation capabilities. The microarray used was a 2240-feature oligonucleotide array which contained 1,152 clones specific to the shrimp. Differentially expressed genes were 1,3-β-D-glucan-binding high-density lipoprotein, 2/3-oxoacyl-CoA thiolase and vitellogenin. With use of this array, the authors saw an increase in expression of the aforementioned genes at later vitellogenic stages (Brady et al. 2012).

Two American lobster studies have been published using microarray technology. They examined immune responses against Aerococcus viridans var. homari and Anophryoides haemophila (Clark et al. 2013a; Clark et al. 2013b). The experiments focused on gene expression in the hepatopancreas which is involved in immune function. These were the first studies to use the American lobster oligonucleotide microarray. The Aerococcus and Anophryoides studies revealed 148 and 145 significant differentially expressed genes respectively (Clark et al. 2013a; Clark et al. 2013b).

These studies pioneered the exploration of crustacean transcriptomic changes in gene expression. With the continued use of microarray technology, expression profiles of the American lobster during different life events, such as reproduction and growth, can be explored more rapidly; however, results must be validated by methods such as RT-qPCR.

1.7 Polymerase Chain Reaction

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was originally designed for the sensitive detection and quantification of specific RNAs (Wang et al. 1989; Bustin 2002; VanGuilder et al. 2008; Pfaffl 2010). Contrary to traditional end point PCR, RT-qPCR allows for the continuous measurement of gene amplification. RT-qPCR has evolved into a method used to measure changes in mRNA expression. Oligonucleotide probes (primers) must be chosen that are both sensitive and specific to the mRNA transcript desired prior to cDNA synthesis. These primers are created from public gene repositories (e.g. GenBank) and expressed sequence tags (ESTs). Primers are created using a variety of software packages which estimate the probability and location of hairpin loops, primer dimers, or self-binding primers. Primer dimers and hairpin loops can have detrimental effects on the efficiency of primer binding and extension, and thus, should be avoided if possible (Derveaux et al. 2010). Having primers which are optimized to the target allows efficiency, specificity, and reproducibility of the results. The analysis tool, qBase^{PLUS}, allows for the specific adjustment to each primer's efficiency. During the optimization process, melt curves are produced which allow the user to identify primers which may not be specific to the target or gene of interest. Melt curves should contain 1 clear peak for each sample. When more than 1 peak is present, a new primer should be sought due to the lack of specificity (Nolan et al. 2006).

Over many thermal cycles, generally between 35 and 40, amplification of product occurs and this is monitored by a change in fluorescence over the course of each cycle. Some common detection methods are 5' nuclease assay TaqMan probes, molecular beacons, and double stranded DNA intercalating dyes. Although there are several detection

methods, they are all able to give an appropriate fluorescence reading. The amplification of product follows an exponential curve that will eventually increase to a point at which the fluorescence of product exceeds that of the background. This is known as the cycle threshold (Ct). The more starting target that there is the lower the Ct will be (Bustin 2002; Ginzinger 2002; Nolan et al. 2006; Wang et al. 2006; VanGuilder et al. 2008).

When compared to microarray analysis, the RT-qPCR method of mRNA expression analysis is low throughput based on the number of genes which can be examined simultaneously. However, this method is currently considered the gold standard for this type of gene expression analysis and microarray validation (Bustin 2002; Morey et al. 2006; Nolan et al. 2006; Wang et al. 2006; Hellemans et al. 2007; VanGuilder et al. 2008). As with microarray reporting, RT-qPCR results require standardization for reporting results and the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were created (Bustin et al. 2009; Bustin et al. 2010; Bustin 2010). The higher sensitivity and specificity to changes in individual gene expression levels are the advantages of this method (Bustin 2002; Morey et al. 2006; Nolan et al. 2006; Wang et al. 2006; Hellemans et al. 2007; VanGuilder et al. 2008).

Vitellogenin of the banana shrimp, Fenneropenaeus merguiensis, was analysed in ovary and hepatopancreas using RT-qPCR analysis. Ovarian expression increased rapidly to stage 2 before decreasing through stage 4 ovaries whereas expression in the hepatopancreas increased steadily to stage 4. These results suggest differential role of intra- and extra-ovarian vitellogenin expression (Phiriyangkul et al. 2007).

Microarrays are validated based on correlation with qPCR, which is affected by the amount and direction of regulation (up- or down-regulation), the array p-value, and the

qPCR p-value. For example, a fold change of at least 1.4 and a p-value of ≤0.0001 will usually return a correlation of at least 0.80 between microarray and qPCR results. Microarrays which do not meet the aforementioned regulation thresholds should not be discarded but rather be treated with caution when proceeding. Correlation is not, however, affected by microarray spot intensity or the use of frozen tissue (Morey et al. 2006). Validations should also be done on highly conserved genes (p-value ≥ 0.9) from microarray experiments for normalization within the PCR software (Derveaux et al. 2010).

Comparisons between microarray and RT-qPCR show the limitations of both methods. Microarrays handle whole genome expression experiments very well but are limited in their precision at a single gene level. RT-qPCR performs well, with increased accuracy, when comparing individual genes but is limited in its ability to compare several genes simultaneously. To examine the same number of genes represented on a microarray, large amounts of biological sample and reagents would be required increasing the price of analysis making it unfeasible, thus a combination of techniques is the most accurate method.

1.8 Rationale

The American lobster fishery is the economic backbone of many coastal communities, although it has not always been such a valuable species. Scientists have studied the American lobster for over a century but as the demand began rising for lobster in the mid to late 1950s, publications began steadily increasing as well. A quick Google Scholar® search for Homarus americanus returned the following steady publication increase (Figure 1.8).

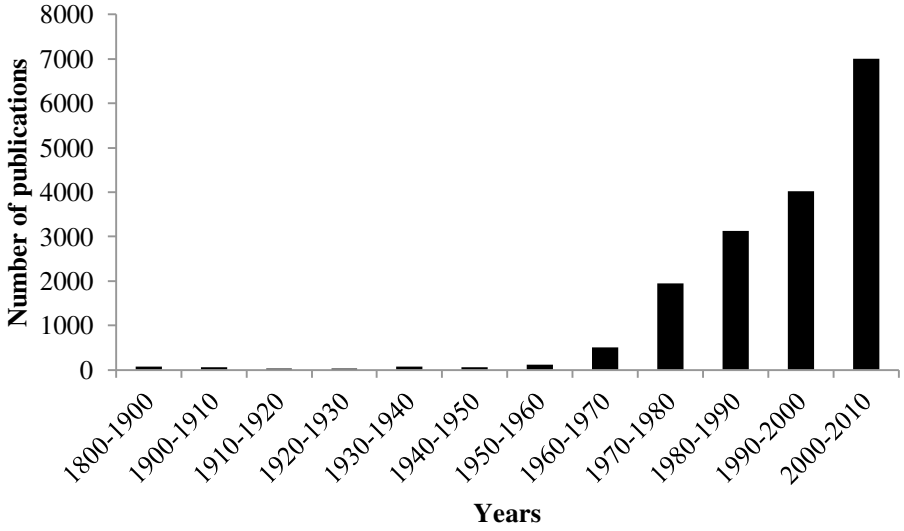

Figure 1.8: Increase in total publications on the American lobster (Homarus americanus) over the past century in Google Scholar®.

Currently, scientists rely on oocyte colour, size, ovary factor, and histology to stage ovaries. Currently, fisheries scientists use lethal methods, such as ovary colour, oocyte size, and ovary factor to determine the SOM_{50}. With the FRCC recommending adjusting the minimum legal size to match the SOM_{50}, to attain the information necessary for each LFA, thousands of female American lobsters need to be sacrificed. Therefore, the development of a universally accepted, non-intrusive ovary staging method is vital. Molecular methods offer a method for exploring potential biomarkers associated with ovary staging by combining high throughput analysis of thousands of genes by microarray with confirmation of gene expression of selected target genes by RT-qPCR. This could be a first step in defining gene targets coding for proteins for future diagnostic development. Also, exploration of thousands of genes gives further insight into molecular mechanisms associated with ovary development and transition during oocyte formation in crustaceans.

Based on the development of ovaries and the physiological changes occurring within the ovary as oocytes mature, I hypothesize that genes controlling reproduction will be differentially expressed at distinct ovary stages correlating with observed morphological ovary stages.

The objectives of this study were to perform the first microarray experiment on female Homarus americanus of differing ovary stages to determine which genes are differentially regulated as female American lobsters reach reproductive commitment. From this, genes could then be identified and classified as either functionally annotated or novel genes associated with reproduction. The genes discovered by microarray experiments are then subsequently validated using RT-qPCR using specific functionally annotated genes of interest (GOI) for American lobster reproduction. These GOI were selected based on their significance in the microarray experiment along with the role of their functional annotation.

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CHAPTER 2: GENE EXPRESSION IN FEMALE AMERICAN LOBSTERS (HOMARUS AMERICANUS) TO DETERMINE REPRODUCTIVE STATUS USING MICROARRAY ANALYSIS OF FOUR TISSUES

2.1 Introduction

American lobster (Homarus americanus, Milne-Edwards 1837) fishing in Canada is the backbone of the economy maintaining many coastal communities. American lobster can be found in the Atlantic Ocean from North Carolina, USA to the southern part of the Labrador Sea (Department of Fisheries and Oceans 2011; Food and Agriculture Organization of the United Nations 2012). In 2011, the American lobster fishery in Canada reached close to $620 million landed value, making it the most valuable Canadian seafood fishery (Department of Fisheries and Oceans 2013). With this economic importance comes the responsibility to manage the industry effectively to maintain sustainability of the American lobster stock. The Fisheries Resource Conservation Council (FRCC) was a non-governmental council composed of representatives from industry and universities that reviewed fisheries at the request of the Minister of the Department of Fisheries and Oceans Canada (DFO) and made recommendations to improve the sustainability of the fishery. The American lobster fishery was reviewed in 1995 and again in 2007. Recommendations were similar in both years in terms of reducing fishing effort and increasing egg production (FRCC 2007). They suggested that one way of increasing the egg production is adjusting the minimum legal size to match the size at which 50% of females are considered mature, referred to as the Size at Onset of 50% Maturity (SOM_{50}) (FRCC 2007).

American lobsters have a hard exoskeleton that must be shed (moult) to grow. Moulting and reproduction are antagonistic, meaning they cannot moult and reproduce at the same time. A female American lobster will mate following its moult with a hard-shell male early in the reproductive cycle. Reproduction typically follows a two-year cycle in which moulting and mating occur in the first summer and egg extrusion happens the second summer hatching the following summer (Nelson et al. 1988; Comeau and Savoie 2002; Waddy and Aiken 2005). American lobsters take ~6 to 8 years to reach sexual maturity although this varies greatly depending on location and water temperature (Comeau and Savoie 2002; Little and Watson III 2005). As the ova develop, the ovaries progress through defined morphological stages (Aiken and Waddy 1980). The ova are extruded onto the pleopods where they are held between 9 and 12 months before releasing stage I larvae.

The seven ovary stages range from immature (stage 1) through developing (stages 2, 3 and 4a) to mature (stages 4b, 5 and 6) and are defined based on a combination of ovary colour (OC), oocyte size (OS), ovary factor (OF), and histology (HIST) (Aiken and Waddy 1980). These methods are used to establish the SOM_{50} within LFAs but are lethal (need to sacrifice the lobster); their application can be subjective prompting debates (Aiken and Waddy 1980; Comeau and Savoie 2002; Waddy and Aiken 2005). Although these methods can result in accurate ovary staging in late spring/early summer, staging females in the fall can be very difficult, especially differentiating between stages 4a and 4b. Determining OC can be subjective and is especially difficult to differentiate among higher stages (stages 4-6). Measuring OS can be difficult due to the large size variation in oocytes located within one ovary. OS should be evaluated based on uniformity of oocyte

size. The largest and smallest oocyte should be measured to determine uniformity (Comeau and Savoie 2002; Waddy and Aiken 2005). Evaluating OF is less subjective although values could overlap depending on the timing of the sampling (Comeau and Savoie 2002). Histology is currently considered the best method for staging ovaries, especially at the critical stages 4a and 4b. One major problem with histological ovary staging is that it is time consuming. Also, the ovary must be placed vertically inside the histology cassette for proper orientation to accurately examine the developing follicles (Michel Comeau, Personal Communication). A major concern with all of these methods is that they require the sacrificing thousands of female American lobsters to examine ovaries with any confidence in determining the SOM_{50} (Comeau and Savoie 2002; Waddy and Aiken 2005).

Investigations into crustacean endocrinology have revealed many associated reproductive factors as techniques have evolved (Laufer 1987; Sarojini et al. 1995; Nagaraju et al. 2003; Tiu et al. 2009; Subramoniam and Kirubagaran 2010). Gonad inhibiting hormone (GIH), gonad stimulating hormone (GSH), and crustacean hyperglycemic hormones (CHH) all play a role in the control of reproduction in decapod crustaceans. These processes are controlled by the X-organ, located in the eyestalk, and the thoracic ganglion, located in the thorax near the brain (Laufer et al. 1991; Chan et al. 2003; Serrano et al. 2004; Fanjul-Moles 2006; Nagaraju 2011)and controls moult processes. Additional to GIH and CHH, the X-organ also produces the moult inhibiting hormone (MIH) which plays essential roles in the control of reproduction and moulting (Chan et al. 2003; Serrano et al. 2004).

Vitellogenin, the egg yolk precursor, is vital to the maturation of developing ovaries. The major source of vitellogenin is the hepatopancreas, where it is secreted into the hemolymph, and binds to the vitellogenin receptor on the surface of oocytes prior to internalization and conversion to vitellin, the egg yolk. The ovary is also thought to produce small amounts of vitellogenin (Tiu et al. 2009). Vitellogenesis is thought to be stimulated by the decreased production of GIH, also known as vitellogenesis inhibiting hormone.

According to Tiu et al. (2009), vitellogenin present in the American lobster is most similar to Cherax quadricarinatus (57%), then Metapenaeus ensis (43%), followed by Charybdis feriatus (38%). This relationship follows the evolutionary relationship within decapod crustaceans. Homarus lobsters are most similar to crayfish which are then closely related to shrimp and crabs. Multiple vitellogenin genes were hypothesized for the American lobster due to the lack of an identical N-terminal amino acid sequence between ovary and hepatopancreas. RT-PCR analysis has also detected slightly different complementary DNA (cDNA) confirming the possibility of multiple vitellogenins within the American lobster (Tiu et al. 2009).

The SOM_{50}, used for setting the minimum legal size, is mostly determined by ovary observation methods. One of the major problems with the methods currently employed is the sacrifice of female American lobsters to gain an accurate assessment to establish the SOM_{50} for the population. This process is time consuming and relies on current ovarian staging methods, which is difficult, especially around stage 4a/4b. Sacrificing these American lobsters removes them from the spawning population eliminating their reproductive capacity. Adjusting the minimum legal size to the SOM_{50} also is difficult,

even within individual LFAs, due to the variation in SOM_{50} at each port (Comeau and Savoie 2002; Little and Watson III 2005; Waddy and Aiken 2005).

Genetic biomarkers for reproduction could create a reliable, universally accepted method for determining ovary staging. One technique that has recently been applied to the American lobster is the monitoring of gene expression analysis by microarray (MA) (Clark et al. 2013a; Clark et al. 2013b). MAs are high throughput allowing simultaneous view of gene expression profiles for thousands of genes. It also allows examination of the interplay among genes during processes, such as reproduction (Karoonuthaisiri et al. 2009; Brady et al. 2012). MA experiments have increased in their applicability to include a variety of crustacean species (Eichner et al. 2008; Karoonuthaisiri et al. 2009; Towle et al. 2011; Clark et al. 2013a; Clark et al. 2013b). With the expanding DNA sequence data from expressed sequence tags (ESTs) and publicly available genome sequence databases, the construction of custom MAs are becoming more common (Tomiuk and Hofmann 2001; Brady et al. 2012; Clark et al. 2013a; Clark et al. 2013b).

Based on the complexity of reproduction, the hypothesis tested in this study was that genes associated with reproduction were differentially regulated at morphologic stages that correlated with ovary stages. The overall purpose of this study was to discover potential biomarkers for Homarus americanus ovary staging using a custom spotted oligonucleotide microarray. Understanding the molecular regulatory changes occurring at reproductive commitment (stage 4a/4b) may lead to the development of better diagnostic tools to evaluate this critical step in ovary development. In the present study, differentially expressed genes were assessed based on their biological relevance through functional annotation of genes available within publicly available repositories. Genes of

interest (GOI) from the microarray were chosen based on their functional annotations being associated with reproduction and validated by RT-qPCR.

2.2 Materials and Methods

2.2.1 Lobster Sampling and Processing

A total of 43 legal size female American lobsters was collected aboard a fishing vessel from Georgetown, PEI in July 2009 and 2010. Carapace length was recorded (mm) and lobsters were tagged for further identification. Lobsters were held in the Atlantic Veterinary College (AVC) Aquatic Animal Facility for upto 5 days at 4°C in individual holding trays in an attempt to reduce stress from handling and transportation. Prior to necropsy, lobsters were assessed for physical health attributes, including weight (gm), tail tone, defensive posture, eyestalk withdrawal, shell hardness, visible lesions or missing appendages and moult staging using pleopod staging (Aiken 1973).

The American lobsters were necropsied (Appendix A) and 12 tissues were collected: heart, ventral nerve cord, eye, antennal gland, ovary, hepatopancreas, claw muscle, tail muscle, intestine/gut, stomach, and gill. Two ovary subsamples were taken and placed in 1G4F (1% Glutaraldehyde:4% Formaldehyde) solution for further analysis. One of these subsamples was used for oocyte size determination (Figure 2.1) while the other sample was processed for histology. Tissues were homogenized with Tri-Reagent (1 mL Tri-Reagent:100 mg tissue) using an OMNI International TH electric homogenizer with a 7 mm rotating stainless steel knife tip (OMNI International, Kennesaw, GA). Between each homogenization the probe was rinsed with 2.5 mL diH_2O and 2.5 mL of fresh Tri-Reagent. Tissues were stored in Tri-Reagent (Appendix B), snap frozen using liquid nitrogen, and stored at -80°C to prevent degradation of sample.

2.2.2 Ovary Staging

Ovaries were staged at the AVCLSC based on ovary colour, oocyte size (Figure 2.1), and ovary factor. Ovary samples for histology were oriented in a tissue cassette and sent to AVC Diagnostic Services for histological slide preparation. Histology slides were sent to DFO Moncton (343 University Avenue) for ovary stage classification but contained poor results, based on orientation and staining methods, and were removed from the staging process.

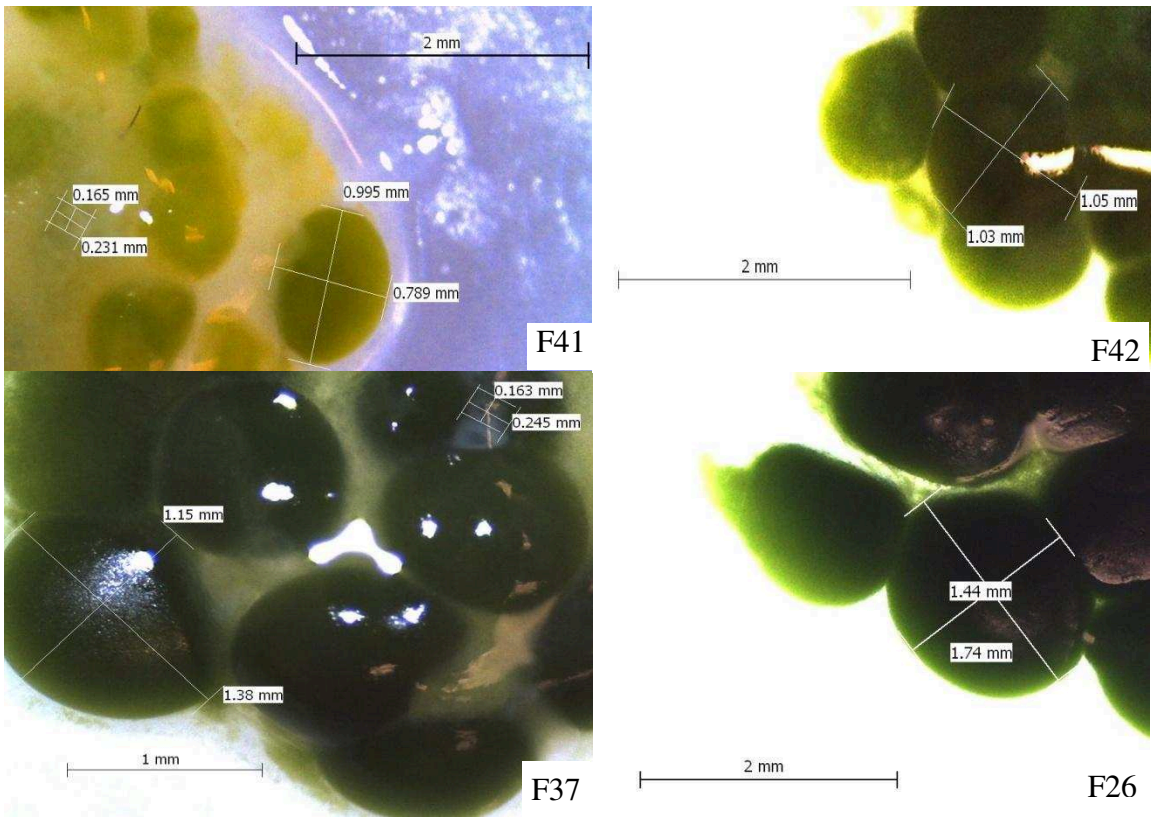

Figure 2.1: Female American lobster (Homarus americanus) oocytes from ovaries. Progressive oocyte colour and size. Largest and smallest oocytes visible were measured to determine oocyte range for ovary staging. F## represent individual female lobsters. Ovary stages were determined using a combination of colour, oocyte size, and ovary factor. Stages are as follows: F41 - ovary stage 2; F42 - ovary stage 3; F37 - ovary stage 4a; F26 - ovary stage 5.

2.2.3 RNA Extraction

RNA was extracted from the ovary, hepatopancreas, and eyestalk, that are all thought to play a role in ovarian-oocyte maturation (Tiu et al. 2008; Nagaraju 2011). RNA was also extracted from haemocyte pellet due to its presence in the hemolymph which transports vitellogenin, and to explore the possibility of a non-lethal biomarker for determining ovary maturation. Once the tissues thawed at room temperature (23°C), 200 µL of chloroform was added for each mL of homogenate. The tissues were then centrifuged at 12,000 x g for 15 min at 4°C using a Beckmann Allegra 25R benchtop centrifuge with a pre-chilled A-10-25 rotor (Beckmann Coulter, Brea, CA). Centrifugation separated the homogenate by density with RNA located in the top aqueous layer. The top aqueous phase (600 µL) was carefully removed as to not disturb the interface. The 600 µL was placed into a new 1.5 mL tube, and an equal amount of 70% ethanol was added. The remainder of the extraction process followed a modified version of the Qiagen RNeasy® Minikits (Qiagen Inc., Valencia, CA) protocol using DNase I digestion to degrade unwanted DNA from isolated RNA samples (Appendix C). The RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) (Appendix D). RNA was measured in triplicate for each sample. The average ng/µL for each sample was considered the true RNA amount. RNA was considered pure if the 260/280 ratio was ≥ 1.9. After every 9 sample reads on the NanoDrop spectrophotometer, nuclease-free H_2O was measured to ensure minimal carry-over. The quality of the extracted RNA was assessed via Bio-Rad® Experion (Bio-Rad, Hercules, CA) (Appendix E). American lobster, like other arthropods, have a 28S rRNA that exists as two smaller fragments, one that overlaps with the 18S rRNA and another that is slightly larger (Towle

and Smith 2006). Therefore the RNA profile and RNA integrity numbers (RIN) produced by Experion were not an accurate portrayal of the integrity of Homarus RNA (Appendix E, Fig. A1). Samples with clear arthropod rRNA peaks and with minimal fluorescence in the fast region were considered intact and were stored at -80°C.

2.2.4 cDNA Synthesis

The four tissues from each American lobster were pooled prior to cDNA synthesis. Ovary, hepatopancreas, and eyestalk tissues were incorporated at 30% each whereas haemocyte pellet was incorporated at 10%. Pooling pre-synthesis ensured uniform cDNA synthesis of the mixture and used less cDNA reagents. The first strand DNA synthesis followed the SuperScript® Plus indirect cDNA labelling system (Invitrogen, Carlsbad, CA) (Appendix F). RNA was centrifuged to 16 µL using a MiVac DNA concentrator (GeneVac, Ipswich, England). 2 µL of Anchored Oligo(dT) primer was added to the total RNA. The RNA was incubated at 70°C for 5 min using a DNA Engine Thermo Cycler (Bio-Rad, Hercules, CA). All cycler protocols used heated lids to reduce condensation unless otherwise specified. Upon completion of annealing, the reaction was placed on ice for a minimum of 1 min prior to adding the master mix which contained 6 µL of 5X first strand buffer, 1.5 µL of 0.1 M DTT and dNTP mix (including aminoallyl modified nucleotides), 1 µL of RNase out, and 2 µL of SuperScript III Reverse Transcriptase 400 U/µL bringing the total reaction volume to 30 µL. This reaction was then incubated at 48°C for 3 hrs. Once cDNA synthesis was completed, excess RNA was degraded using 15 µL of 1 N NaOH and incubated at 70°C for 10 min, after which 15 µL of 1 M HCl was added to neutralize the reaction. Spin columns provided with the kits were used to purify the reaction mixture. 30 µL of DEPC water was used to elute the cDNA. cDNA

quantity was determined using a 1 µL:4 µL cDNA:nuclcease-free H_2O with a NanoDrop spectrophotometer. The remaining cDNA was stored at -80°C until use.

2.2.5 Pooled Reference Makeup

1 American lobster from ovary stages 2, 3, 4a, and 4b were randomly chosen by use of a random number generator to be used as a reference sample. This was expected to most accurately represent the sample population and placed focus around the change in female American lobsters from 4a to 4b. Individual tissue RNA was combined prior to amplification in identical concentrations to cDNA synthesis concentrations. The combination of these tissues was then added to 1 tube and mixed thoroughly. The mixture was speed-vacuumed to a final volume of 10 µL. The amplification was carried out using in vitro transcription (IVT). The IVT reaction followed the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit protocol (Ambion, Austin, TX) (Appendix G).

One µg of total RNA was used to create first strand cDNA. Synthesis of first strand cDNA created a single 20 µL reaction with the following: 10 µL of total RNA, 1 µL of nuclease free H_2O, 1 µL of T_7 oligo(dT) primer, 2 µL of 10X first strand buffer, 4 µL of dNTP mix, 1 µL of RNase inhibitor, and 1 µL of ArrayScript (RT). T_7 oligo(dT) primer includes a T_7 promoter on the 5' end of the primer sequence with a poly-T region on the 3' end. After combining all components, the reaction was incubated at 42°C for 2 hrs. Upon completion, second strand synthesis was completed bringing the reaction volume to 100 µL. Second strand synthesis combined the following: 63 µL of nuclease free water, 10 µL of 10X second strand buffer, 4 µL of dNTP mix, 2 µL of DNA polymerase, 1 µL of RNase H, and the 20 µL first strand reaction. This mixture was incubated for 2 hrs at

16°C without a heated lid. The reaction was purified from residual reaction components and degraded RNA via spin columns and purification methods supplied with the amplification kit.

A master mix containing 3 µL of 50 mM aminoallyl-UTP (aaUTP), 12 µL of ATP/CTP/GTP mix, 3 µL of UTP (50mM) 4 µL of T_7 10X reaction buffer, and 4 µL of T_7 enzyme mix was added to the double strand cDNA. This brought the total mixture volume to 42 µL. The IVT amplification proceeded over 14 hrs at 37°C, repeatedly transcribing the second strand cDNA. After the 14 hour amplification, the reaction was stopped by adding 58 µL of nuclease free water. Amplified RNA (aRNA) was purified using provided spin columns. aRNA was eluted off using nuclease free water. Upon completion of aRNA amplification and purification, the samples were drawn and diluted 5x using nuclease free H_2O for NanoDrop quantification. The reference sample was then aliquoted into 6 µg amounts and stored at -80°C.

2.2.6 cDNA and aRNA Labelling

cDNA and aRNA differential labelling occurred immediately prior to hybridization onto microarrays. Lights were dimmed during the labelling of cDNA and aRNA to minimize photobleaching due to the sensitivity of the dyes to light.

cDNA labelling followed the Invitrogen™ SuperScript™ Plus Indirect cDNA Labelling System handbook (Invitrogen, Carlsbad, CA) (Appendix H). cDNA was thawed at room temperature (23°C). First strand cDNA was then dried to less than 3 µL using the Genevac speed vacuum. 5 µL of 2X coupling buffer were added to dried cDNA prior to adding AlexaFluor Dye 555. The dye (60 µg) was reconstituted in 2 µg of room temperature (23°C) DMSO, vortexed, and added to the cDNA mixture. The mixture was

wrapped in tinfoil and placed in a dark drawer for 2 hrs at room temperature (23°C). Labelled cDNA was purified using provided spin columns. The final labelled cDNA was quantified using the microarray function of the NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Fluorescence was assessed using this function. Wavelengths measured were 260 nm, 550 nm, and 650 nm to determine dye fluorescence per microgram of cDNA by use of the NanoDrop 1000. The labelled cDNA was carried through when the concentration (pmol/µL) of AlexaFluor® 555 Reactive Dye was higher than AlexaFluor® 647 Reactive Dye.

aRNA was labelled with AlexaFluor® 647 Reactive Dye. The protocol followed that of the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Austin, TX) (Appendix I). aRNA aliquots were dried down to less than 0.5 µL, 9 µL of coupling buffer was added to the dried aRNA. 11 µL of DMSO was added to the dye and vortexed. The aRNA/coupling buffer mixture and suspended dye solution were combined and incubated in the dark for 2 hrs at 23°C. The labelled aRNA was purified using the spin column method by MEGA clear (Ambion, Austin, TX). Purified labelled aRNA was eluted using nuclease free water heated to 55°C. The final labelled aRNA was quantified using the microarray function of the NanoDrop 1000 spectrophotometer described previously. The labelled aRNA was carried through when the concentration (pmol/µL) of AlexaFluor® 647 Reactive Dye was higher than AlexaFluor® 555 Reactive Dye. Remaining labelled aRNA was stored in the dark at 4°C until use later that day.

2.2.7 Microarray Design and Construction

The design of the microarrays was based on publicly available gene sequences and ESTs determined from cDNA of multiple tissues from male and female American lobsters with

multiple physiological conditions (Towle and Greenwood, Unpublished). A total of 29,636 American lobster ESTs was available in dbEST (www.ncbi.nlm.nih.gov/genbank). A total of 15,864 unique EST sequences was discovered using CLOBB and Gene Indices Clustering Software (TIGR) (Parkinson et al. 2002; Pertea et al. 2003). Functional annotation of each EST was done manually by determining the most informative gene ontology revealed by BlastX through Blast2Go (Conesa et al. 2005). The American lobster microarray was constructed of 50 mer probes designed using Array Designer 4 software (Premier Biosoft International). 14,592 probes were produced which contained high binding specificity, GC content, and annealing temperature (Towle and Greenwood, unpublished). Unique identification numbers (HAZ####) were assigned to each probe which acted as an identifier throughout this study. Probes were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA) and submitted to the Vancouver Prostate Centre DNA microarray facility in 250 µM aqueous solutions. Oligonucleotide probes were printed on Erie C28 Aminosaline coated glass slides (2 arrays/slide) with a QArraymax arrayer. There were 14,592 American lobster specific sequences, 210 Sigma Alien DNA controls, 80 GFP controls, 78 buffer controls, and 416 empty controls for a total of 15,376 spots. Quality of printing was monitored using a 9-mer hybridization GenePix 4200AL and visualized with Imagene version 8.0.1 at the Vancouver Prostate Centre prior to arriving at the AVC Lobster Science Centre (AVCLSC) for use.

2.2.8 Hybridization Preparation

Hybridizations each required 100 picomoles of labelled aRNA and 140 ng of labelled cDNA. Upon determination of required amounts, the labelled aRNA required

fragmentation for incorporation onto oligonucleotide microarrays. aRNA was fragmented using the Ambion Fragmentation Kit protocol (Ambion, Austin, TX) (Appendix J). aRNA volume was brought to a working volume of 9 µL with nuclease free H_2O. 1 µL of 10X fragmentation buffer was added to the aRNA. The reaction was incubated at 70°C for 10 min, after which 1µL of 200mM EDTA pH 8.0 stop solution was added. The required amount (µL) of aRNA was multiplied by 11/9 to account for the added liquid from the fragmentation procedure. The fragmented aRNA was then stored on ice until use.

The hybridization mixture used was a modified version of that employed by the University of Victoria (Appendix K). A total of 62.5 µL mixture was used for hybridization. 50 µL of Hyb solution 3 (Ambion, Austin, TX), 1 µL of LNA Blocker (Ambion, Austin, TX), 11.5 µL of DEPC treated water containing labelled aRNA and cDNA were mixed for hybridization. The hybridization mixture was denatured at 80°C for 10 min prior to injection. The mixture was injected at 65°C.

2.2.9 Hybridization

Thirty-two American lobsters were used for hybridization and analysis (Appendix R). Hybridizations were with Tecan HS 400 Pro Dual Area Hybridization chambers (Tecan, Männedorf, Switzerland). Hybridization buffers used during washing sequences were created prior to beginning hybridization. Channel 1 contained 5X Saline-Sodium Citrate (SSC) buffer, 0.01 % SDS, and 0.2% Bovine Serum Albumin (BSA); Channel 2 contained 2X SSC and 0.2% SDS buffers; Channel 3 contained 0.2X SSC buffer; Channel 4 contained 5X SSC buffer; Channel 5 contained 0.2X SSC and 0.2% SDS buffer; and Channel 6 contained only deionized water. Channel 1 was used to prime the

hybridization chamber for 30 sec prior to all hybridizations. The hybridization ran overnight (~16 hrs) using identical conditions on each array (Appendix K). Once completed, each glass slide was carefully rinsed with deionized H_2O to remove any lingering hybridization mixture. Each chamber was rinsed with 0.2% SDS, 30% ethanol, and diH_2O before air drying in a photosensitive environment.

2.2.10 Array Scanning

Each array was then scanned using the Axon GenePix® Pro 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA). A preview scan was done to orient the scan area. Auto photomultiplier tube (PMT) was used to balance channels for acquiring the image. Arrays were saved as .tiff files for further analysis. The files were then imported into SpotReader (Niles Scientific, Portola Valley, CA) and aligned to a GenePix Array List (GAL) file to determine the exact location of spots and label each spot with the best hit from GenBank. Each spot was subjected to a list of pre-set flagging parameters (Appendix L). Spots which fell outside the specified criteria were flagged and excluded from further analysis. Further analysis was performed using a combination of Acuity® 4 (Molecular Devices, Sunnyvale, CA) and TM4 MultiExperiment Viewer (MeV) (Saeed et al. 2003).

2.2.11 Array Analysis

The microarrays were normalized using LOWESS normalization. Once normalized, a dataset was created for spots on the array hybridization image that met the following criteria. Each spot had to have a signal to noise ratio (SNR) of 3 or greater in both red and green spectra. Each spot also had to be flagged as 'good' to be included. Then, based on the number of returned genes, microarrays were either included or excluded based on a

60% cut-off. Each microarray was then classified and sorted based on ovary stages. To determine the number of times a certain gene was represented in each of the stages, a count was performed on each gene for each stage. Genes were removed if they were present in less than 70% of each ovary stage. These cut-offs maintained a majority of microarrays and statistical significance within each ovary stage. Microarrays were grouped into respective ovary stages prior to performing a one-way ANOVA. The cut-off was at $p \leq 0.05$ and all genes with $p > 0.05$ were removed and analyses were done on the remaining significant genes. Hierarchical clustering was used in the analysis process due to simplicity and the ability to group both microarrays and genes based on gene expression profiles. This was depicted by a hierarchical tree and heat map. For the tree, microarrays which were most similar grouped closest together and those with the largest difference grouped furthest apart. The same was true for the grouping of genes. K-means analysis was also done to group genes into clusters with similar expression profiles. Figure of merit (FOM) was employed prior to clustering to determine the optimal number of K-means clusters. ANOVA data and gene expression profiles were exported to Microsoft Excel for further analysis. Genes with $p \leq 0.05$ were used for further analysis. EST sequences were imported into Blast2GO (Consesa et al. 2005) where each sequence was screened against publicly available sequences in NCBI databases. Sequences were then mapped and functionally annotated. Genes with best hits that were related to reproduction or growth were then selected for further analysis and validation. Genes of interest (GOI) were selected based on their best hit and proposed function. Highly conserved (reference) genes were chosen based on the largest p-values, not on function.

2.2.12 Reference Gene Selection

RT-qPCR reference genes were selected from the list of highly conserved genes. Reference genes required $p > 0.5$, differential expression of ≤ 2, and a standard deviation of $< 30\%$. Selected primers were in the top 20 highly conserved genes based on 1-way ANOVA results instead of functional annotation. A total of 7 genes was selected for further analysis.

2.2.13 RT-qPCR Genes of Interest Selection

GOI were selected based on functional annotation. Genes with $p \leq 0.05$ were examined. Genes without functional annotation or hypothetical protein function were removed from further analysis. GOI had predicted functional annotation of either reproduction or growth. In total, 15 genes were prioritized based on the aforementioned criteria.

2.2.14 RT-qPCR Primer Creation

The primers for the GOI and reference genes were constructed based on best hit EST sequences and selected for areas which did not contain secondary structures, namely hairpin loops, at 60°C with 50 mM monovalent cations, 5 mM divalent cations and 200 mM primer concentrations, to enhance the amplification efficiency. Monovalent and divalent cations were chosen from pre-existing AVCLSC laboratory protocols, where as primer concentration was determined from the Invitrogen (Carlsbad, CA) MEGA RT-qPCR protocol. Primer design was completed by using multiple software packages, including DINAmelt server (mfold.rna.albany.edu/?q=DINAMelt/Two-state-folding), Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), and NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) (Appendix M). The use of

these software packages ensured that the created primers were specific to the associated genes. Primers were then obtained from Integrated DNA Technologies (Coralville, Iowa).

2.2.15 Reverse Transcription for qPCR

All experimental components of RT-qPCR followed the MIQE guidelines throughout (Bustin et al. 2009; Bustin et al. 2010; Bustin 2010). Prior to optimization, stored RNA was thawed and reverse transcribed into cDNA using SuperScript III® RT (Invitrogen, Carlsbad, CA). 1 µg of total RNA (300 ng of ovary, hepatopancreas, eyestalk, and 100 ng of haemocyte pellet) was added to 1 µL of oligo(dT) primer (50 mM), 1 µL of annealing buffer, and the entire volume was brought up to 8 µL using DEPC treated water. The mixture was incubated for 5 min at 65°C after which the mix was placed on ice for at least 1 min. Reverse transcription reaction components were 10 µL of 2X first strand reaction mix and 2 µL of Superscript III/RNase out enzyme mix (Appendix N). During one reverse transcription reaction, 2 µL of nuclease-free water replaced 2 µL of Superscript III/RNaseOUT enzyme mix to serve as a negative control for RT-qPCR analysis. The reverse transcription reaction was incubated at 50°C for 50 min and the reaction was stopped by heating to 85°C for 5 min. Once terminated, 40 µL of DEPC treated water was used to dilute the reaction. The final concentration was aliquoted 30x with a volume of 2 µL each.

2.2.16 Primer Optimization Conditions and Analysis

Upon arrival of primers, they were optimized and deemed efficient. Primers were sent lyophilized and were reconstituted. Primers were first spun at 5,000 x g at 23°C for 2 min and then diluted 1:10 µmol using nuclease-free H_2O. With the use of a Chromo4 fluorescence reader and DNA Engine thermocycler (Bio-Rad, Hercules, CA), all primers

were assessed on a gradient using a three step annealing procedure. Opticon Monitor 3 (Bio-Rad, Hercules, CA) was used to read the fluorescence of amplification reactions. The gradient was run from 55°C to 70°C over 12 wells. Specific temperatures were determined using the Opticon Monitor 3 gradient calculator. Reactions were run in either 0.2 mL low profile tubes with clear caps or 0.2 mL low profile 96-well plates sealed with microseal 'B' seals (Bio-Rad, Hercules, CA). Optimization reactions were performed in 20 µL samples. These reactions consisted of 10 µL of EXPRESS SYBR® GreenER™ qPCR Supermix with premixed ROX, 0.4 µL 10 µM forward primer (200 nM final), 0.4 µL 10 µM reverse primer (200 nM final), 6.7 µL DEPC H2O and 2 µL cDNA template (Appendix O). Each primer set was analyzed individually; each primer had its own optimum annealing temperature and specificity of the reaction. Melt curves were produced using Opticon Monitor 3 software to determine the specificity of the primer set to the sample. Generally, a single peak above 80°C ensures specificity. The primer with the lowest cycle threshold value (C_t) at the highest temperature was the most specific. Products were then run on a 1.5% agarose gel electrophoresis at 90 volts ensuring production of a single DNA band being produced from each primer set (Appendix P).

Efficiency was determined with 5-fold standard curve with points at 5^0, 5^{-1}, 5^{-2} template concentrations. Each dilution point was run in triplicate technical replicates, meaning the same sample was run 3 times. The standard curve C_t values were plotted against the logarithm of the 5-fold dilution factor. A linear regression line was plotted on each graph. The amplification efficiency was determined by the equation: Efficiency = $10^{(-1/\text{slope of the regression})}$. This was converted to percent efficiency for simplicity by the equation: % Efficiency = $(10^{(-1/\text{slope of the regression})}-1)*100$. Primers had to be 90-105% efficient with an

R^2 for the regression line of at least 98%. Technical replicates were required to be within 5% of each other to ensure repeatability. Efficient primer sequences were recorded from 5' to 3' (Table 2.1).

Table 2.1: Three step qPCR reactions were used for primer efficiencies with melt temperatures (Tm) below 62°C. Three step reactions began with 2 min incubation at 50°C, increasing to 2 min incubation at 95°C, then 40 cycles of: 5 sec at 95°C; 20 sec at primer specific Tm for annealing; 20 sec at 72°C for elongation. A fluorescence measurement was taken after each elongation step. A melt curve was performed from 65°C – 90°C with fluorescent reads every 0.5°C for 2 sec. HAZ numbers and GenBank accession numbers are listed for each gene.

HAZ number	Protein Name (GenBank accession number)	Melt Temp (°C)	Amplicon (bp)	Efficiency (%)	Primer Name	Primer Sequences (5'-3')
Genes of Interest (Template)						
HAZ09100	Inhibitor of growth protein 1-like (EH401275)	55	80	102.6	T100F2	AAGTGACCTATGAGTGTTAG
					T100R2	GTAGCTGAGTATAATGCTTC
HAZ09211	Egg-derived tyrosine phosphatase (FD699965)	55	112	98.2	T211F2	CAGAGCAGAGCATTATAG
					T211R2	GAGCAAAGTTAGAGACTAGG
HAZ00274	Growth-hormone inducible transmembrane protein (FD584754)	56.3	208	95.5	T274F2	CCGAGACACTTACTCATAC
					T274R2	TAACACACCACAGTTGAC
HAZ11280	97 kDa heat shock protein (EX486935)	57.5	302	97.2	T280F2	CCATCTACAACTCTTAACC
					T280R2	CTTTTGACACTCTCCTTC
HAZ08352	Quaking protein A (EH116194)	57.5	118	101.2	T352F1	TAGAAGACACAGAGAATAGG
					T352R1	CTGGAAGAATCTCTGTATG
HAZ10408	HaVg3 (EW997929)	55.4	227	99.6	T408F2	TCTTCCTGGAGAGTAATAG
					T408R2	GACACCACTCTGAAGAAC
HAZ17438	Ovary development-related protein (FE659902)	55	177	104.0	T438F1	GGGATACATAGACGATTG
					T438R1	GTCATTCTTGAAGGTAGTG
HAZ14456	HaVg4 (FD467667)	55.4	136	98.8	T456F1	CCTTGCTAGAGTACAAAG
					T456R1	TCTAGTTGTTCTATCAGACG
HAZ01770	Ovary development-related protein (FD584577)	55	178	101.1	T770F1	AGGTGGTGTTCAATCTAC
					T770R1	GTTTACAACAGTCGTCTATG
HAZ16854	Estradiol-17-β-dehydrogenase 12-B-like (FE535321)	55.4	206	99.2	T854F2	GAAGATTGTGTTAGTCAGTC
					T854R2	GCTGTTATACGTCTTCTG
HAZ07899	HaVg1 (EF422415.1)	57.5	143	99.5	T899F2	GTAGTTAGCACACAGGAAC
					T899R2	GAACTCTTTGGTAGTAACTG
HAZ11967	HaVg2 (EX568231)	57.5	179	97.6	T967F2	GTACAGCAGAACATCAAG
					T967R2	ATAGACTGGGGTTAGTTTC
Reference Genes						
HAZ10762	Reference 1 (unannotated) (EX471325)	55.4	190	94.2	R762F1	ACTCTCTCTCTCTCTCTTTC
					R762R1	TAGGTCTTCTGATAGGTCTC
HAZ12968	Reference 2 (unannotated) (EY290744)	56.3	79	92.9	R968F2	GGAGTTCCTCGTTTTACTAC
					R968R2	TAACACAGGAAACAGGTC
HAZ02982	Reference 3 (unannotated) (CN951221)	56.3	192	93.7	R982F2	GACCTGAAATACCTTGAC
					R982R2	AGAAGCAGTACCTCATTG

2.2.17 RT-qPCR

Once the reaction efficacy for each gene was optimized, RT-qPCR was done using the Chromo4 and DNA Engine thermal cycler (Bio-Rad, Hercules, CA) (Appendix Q). There were 24 American lobsters used for RT-qPCR analysis (Appendix R). To reduce human error in the setup of the experiment, samples and master mix were pipetted into 0.2 mL low profile 96-well plate (Bio-Rad, Hercules, CA) using the QIAgility automated PCR setup machine (Qiagen, Valencia, CA) and were manually sealed using the microseal 'B' seals (Bio-Rad, Hercules, CA). Total reaction quantities were reduced from 20 µL, as in optimization, to 15 µL working volume in each well to reduce the quantity of master mix required. Each plate contained all samples in triplicate and included triplicate no template controls (NTC) and a no reverse transcriptase (enzyme mix) controls (No-RT) to test for nucleic acid contaminants in the reaction. All samples, including both controls, were run on 1 96-well plate per gene which eliminated inter-run differences.

2.2.18 Reference Gene Stability

qPCR raw data was imported into qBase^{PLUS} (Biogazelle, Zwijnaarde, Belgium). Only 3 reference genes from the 7 ordered reference genes passed optimization and efficiency calculations to be viable to perform RT-qPCR. These 3 genes were analyzed by use of GeNorm analysis in qBase^{PLUS}. GeNorm M values of < 0.5 and $V < 0.15$ were considered stable. GOI were also imported for GeNorm analysis, due to minimal reference targets and low expression of a few GOI.

2.2.19 Analysis of qPCR Data

Average efficiencies were then entered for each sample, adjusting all downstream analysis to the target efficiency. qBase^{PLUS} quality control settings remained the default settings of C_t range for technical replicates of ≤ 0.05 C_t and the minimum distance between highest C_t and negative control of at least 5 C_t (Hellemans et al. 2007).

Normalization was done using the three most stable reference genes. Since all tissue samples were run on 1 plate for each gene, no interrun calibration was required. Therefore, the calibrated normalized relative quantities (CNRQ) did not differ from the original normalized values. Values were scaled to average expression levels to monitor the genetic regulation over ovary stages. Changes in relative expression, therefore, represent a differentiation from the average expression level. Results were imported into SPSS for further statistical analysis. All data was $\log_2$ transformed. First, the Shapiro-Wilk test for normality was used to test for normality, than an ANOVA was done with a Benjamini post-hoc multiple test correction. Non-normal data were analyzed using the Kruskal-Wallis test statistic. In all statistical tests, significance was determined at $p \leq 0.05$.

2.2.20 Vitellogenin Sequence Comparisons

Percent similarity of gene alignments was done using LALIGN (Swiss Institute of Bioinformatics, Lausanne, Switzerland) for nucleotide and NCBI-BLAST (blast.ncbi.nlm.nih.gov) for proteins. All ESTs represented were translated to protein with 6 open reading frames (3 forward and 3 reverse frames) using EBI's EMBOSS Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq). All 6 translated protein open reading frames were compared to HaVg1 and the highest similarity was used for

protein similarity levels. To show the similarity among genes, two unrooted neighour-joining phylogenetic trees, one at the nucleotide level and one at the protein level, were created using a bootstrap value of 1,000 using MEGA5 (Tamura et al. 2011).

2.3 Results

2.3.1 Microarray Results

RNA extracted from four tissues (ovary, hepatopancreas, eyestalk, and haemocyte pellet) was combined and tested using dual channel microarray experiments for five ovary stages (2, 3, 4a, 4b, and 5). A pooled reference design was employed to compare expression levels over ovary stages. After flagging had taken place, there were 7,716 suitable genes remaining. Among these genes only 2,709 (37.2%) of genes were functionally annotated. Although morphological ovary staging was done, hierarchical clustering was used to determine which female American lobsters grouped into which ovary stage, especially stages 4a and 4b (Figure 2.2) because some of the stage 4 lobsters were unclassified (i.e. no decision could be made based on morphological ovary staging). From the hierarchical clustering, lobster F22 was determined to be stage 4b whereas lobsters F15 and F37 were grouped with the stage 4a lobsters. Figure of merit, used prior to k-means clustering, determined the optimum number clusters to be 5.

Cluster 1 contained a total of 276 statistically significant genes which represents 16% of all genes represented in clusters. Of these genes, 155 had hits within GenBank but only 144 of these genes were functionally annotated as determined by Blast2Go. The average expression profile for this cluster indicated an upregulation to stage 3 before downregulation at stage 5 (Figure 2.3). Cluster 1 contained genes which coded for reproduction and growth, such as estradiol 17-beta-dehydrogenase, farnesoic acid o-methyltransferase, growth hormone inducible transmembrane, inhibitor of growth protein 1, and ovary development-related protein (Appendix S; Table S1). This cluster was dominated by genes dealing with cellular (n=76), developmental (n=30), metabolic

(n=63), multicellular organismal processes (n=31). Also included in large proportion were genes coding for biological regulation (n=39), response to stimulus (n=29), and localization (n=27).

Cluster 2 contained 251 statistically significant genes which represented 14% of genes within clusters. There were 67 genes which had GenBank hits but only 62 were functionally annotated. This cluster contained downregulated expression values across all ovary stages. Expression decreased to stage 3 before increasing in expression to stage 5 (Figure 2.3). Cluster 2 contained genes whose function coded primarily for cellular (n=20) and metabolic processes (n=18). Other functions represented were response to stimulus (n=10), biological regulation (n=9), biogenesis (n=8), and localization (n=8). In this cluster there were few reproductive related genes, 97 kDa heat shock protein, which is an egg-sperm receptor, was one gene chosen for further analysis (Appendix S; Table S2).

Cluster 3 had 548 statistically significant genes which represented 31% of clustered genes. Only 189 genes contained hits within GenBank. Of these genes, 160 genes were functionally annotated. This cluster contained downregulated values for ovary stages 2, 3, and 4a before becoming upregulated in stages 4b and 5 (Figure 2.3). Reproductive related genes in this cluster were vitellogenin (HaVg3), ovary development related protein, and egg-derived tyrosine phosphatase. Genes coding for development and/or growth consisted of ecdysone-inducible proteins, cuticle proteins, and an innexin-like gene (Appendix S; Table S3). Cellular processes and metabolic processes dominated functional annotation at n=85 and n=81, respectively. Also represented were biological

regulation (n=35), biogenesis and localization (both n=25), response to stimulus (n=22), development (n=21), and multicellular organismal processes (n=20).

Cluster 4 contained 517 statistically significant genes and represented 29% of clustered genes. Of these, there were 160 genes with hits in GenBank and 143 of these were functionally annotated. This cluster remained downregulated over all ovary stages. The average profile of this cluster decreased in expression through ovary stage 4a before increasing at stage 4b and increased further at stage 5 (Figure 2.3). This cluster contained many genes relating to development and reproduction. Some genes coded for H. americanus vitellogenin genes (HaVg1 and HaVg2), a lola protein, a cuticle protein, quaking protein, and innexin 3 (Appendix S; Table S4). Metabolic processes (n=64) and cellular processes (n=60) both dominated this cluster. Other annotations represented were response to stimulus (n=24), biological regulation (n=23), localization (n=18), multicellular organismal processes (n=16), and biogenesis and development (both n=13).

Cluster 5 contained 182 or 10% of statistically significant genes. Of these, 69 genes were represented by hits within GenBank but only 60 of these were functionally annotated. This cluster had positive expression represented by general upregulation from ovary stages 2 through 5. Expression levels seem to hold steady between ovary stages 3 and 4a before increasing relatively linearly in ovary stages 4b and 5 (Figure 2.3). In this cluster there was one vitellogenin gene (HaVg4) and a dopamine beta-hydroxylase gene. There were also two development related genes present (Appendix S; Table S5). Cellular processes (n=26) and metabolic processes (n=21) represented the majority of functional annotation. Biogenesis (n=11), localization (n=8), biological regulation (n=6),

multicellular organismal processes (n=5), and response to stimulus and development (both n=4) were also represented within this cluster.

Vitellogenin, the yolk precursor, was differentially expressed over several ovary stages. There were four variants of vitellogenin genes differentially expressed during the study. HaVg1 and HaVg2 were most similar with HaVg4 being the next most similar and HaVg3 being the least similar of all four Homarus vitellogenins (Figure 2.4). The first differentially expressed vitellogenin was from Homarus americanus. This gene was the complete mRNA sequence retrieved from GenBank using a nucleotide BLAST. This gene was determined to be HaVg1 (Tiu et al., 2009). Since this was the only complete mRNA/protein for Homarus americanus, it was used as a reference for aligning other vitellogenin genes.

An EST most similar to HaVg1 was differentially expressed over the American lobster's ovary stages. This gene was 85% similar to HaVg1 at the nucleotide level and 74% similar at the protein level. With the dissimilarity of the protein sequence, this gene was assigned the abbreviated name HaVg2 following the naming convention of Tiu et al. (2009).

Another vitellogenin EST, most similar to Cherax quadricarinatus, was differentially expressed during the study. This gene was 55% similar to HaVg1 at the nucleotide level and the protein level. Due to the similarity at both levels, this gene was given the conventionally abbreviated name HaVg3.

The last of the differentially expressed vitellogenin genes was one most similar to vitellogenin in Metapenaeus ensis. This gene was 65% similar to HaVg1 at the nucleotide

level and at the protein level, it was 44% similar. This gene was assigned the conventionally abbreviated name HaVg4.

Overall, 569 statistically significant genes were functionally annotated. This represented ~32% of all functionally annotated statistically significant genes. Cluster 1 had the highest functional annotation while cluster 2 had the lowest functional annotation of all clusters at 52% and 25% of genes being functionally annotated, respectively. Clusters 3, 4 and 5 had 29%, 28% and 33% of genes functionally annotated, respectively.

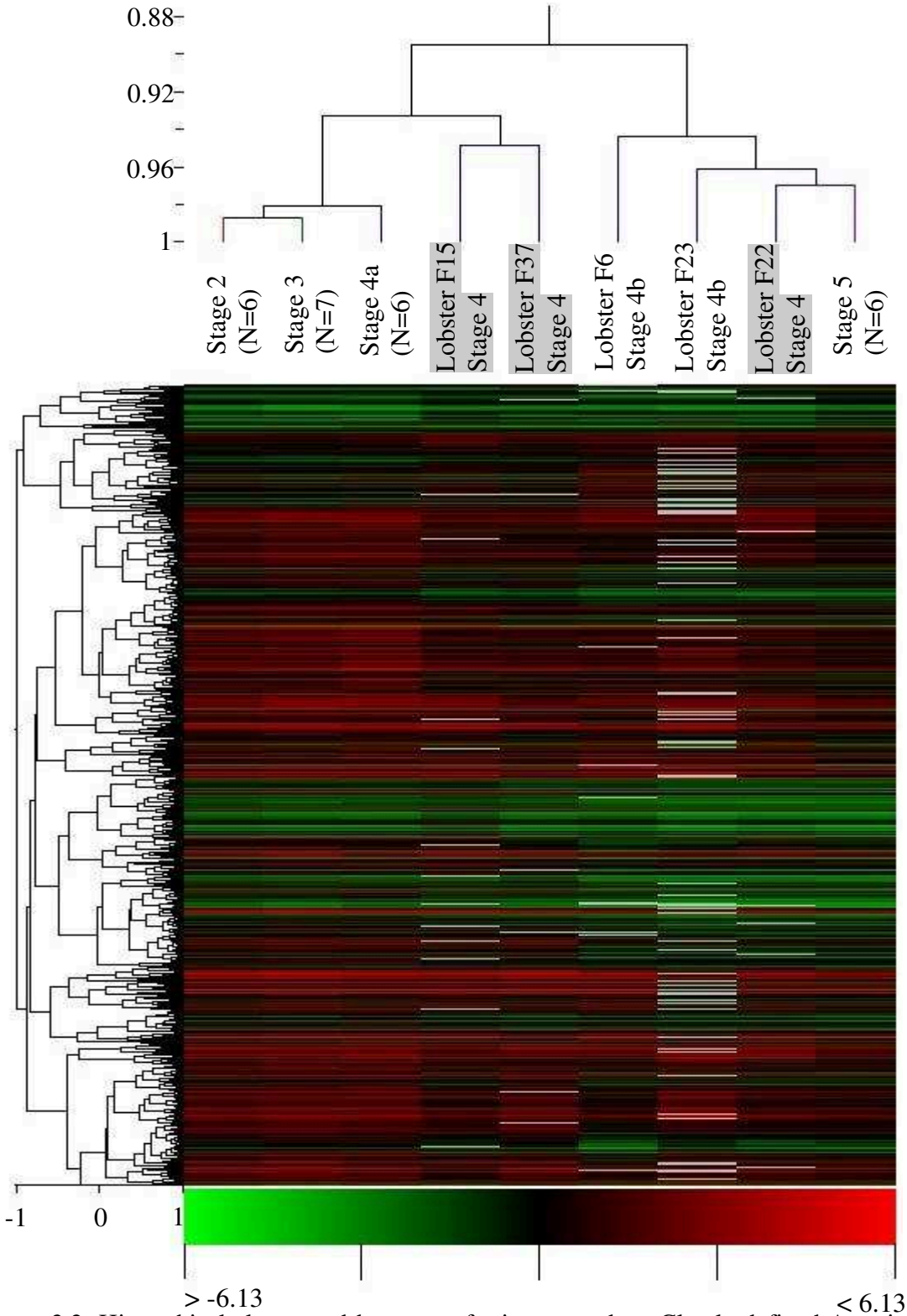

Figure 2.2: Hierarchical cluster and heatmap of microarray data. Clearly defined American lobsters (Homarus americanus) with ovary stage 2, 3, 4a, or 5 by current ovary staging methods were grouped together, while those staged at 4b or unable to be classified using current methods were left separate. Unclassified stage 4 lobsters are highlighted in grey.

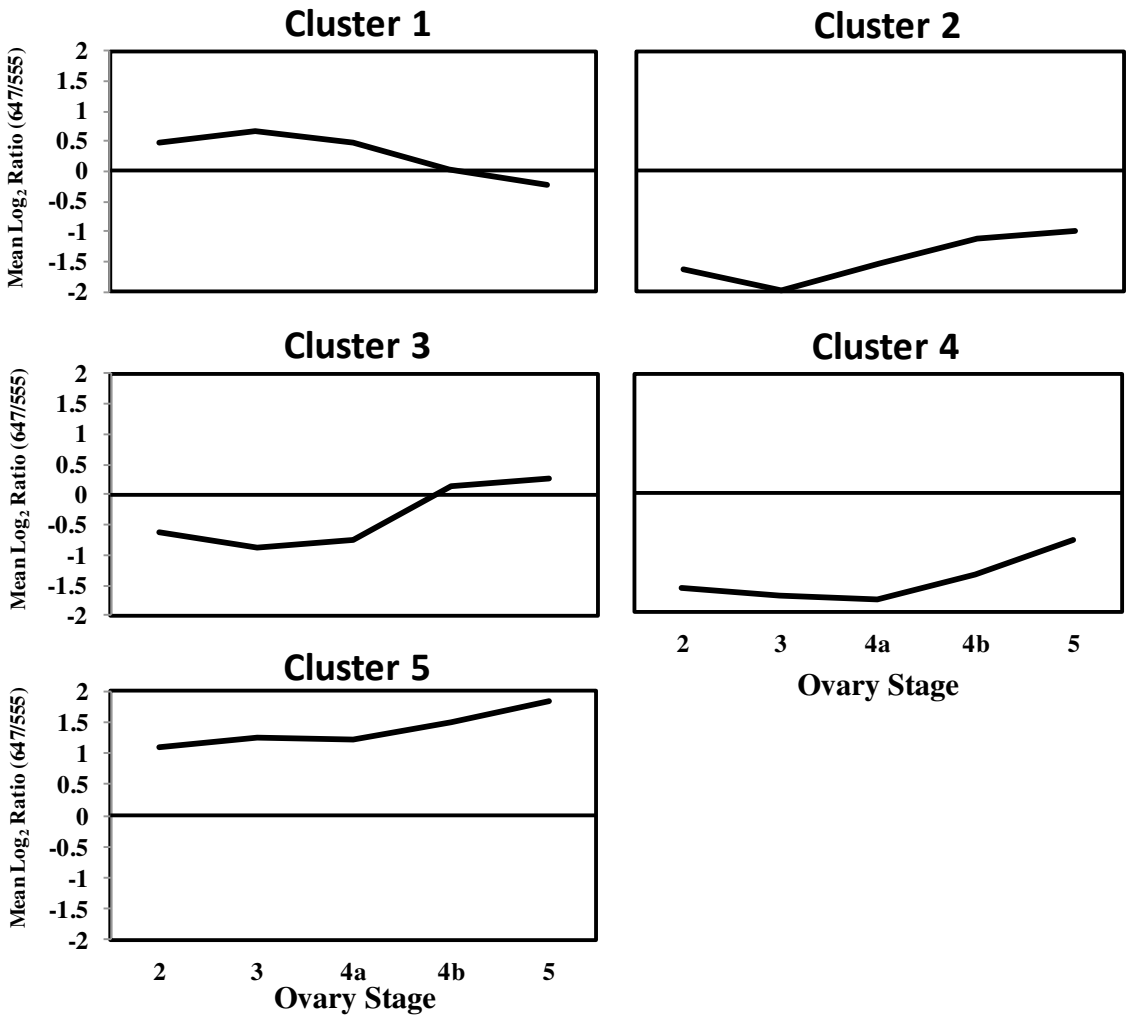

Figure 2.3: Averaged expression profile of k-means clusters 1-5. Data points represent the averaged Log_2 ratio (647/555). Each cluster number was represented above each panel. The inverse of original ratios was taken; positive values corresponded to an upregulation and negative values corresponded to a downregulation.

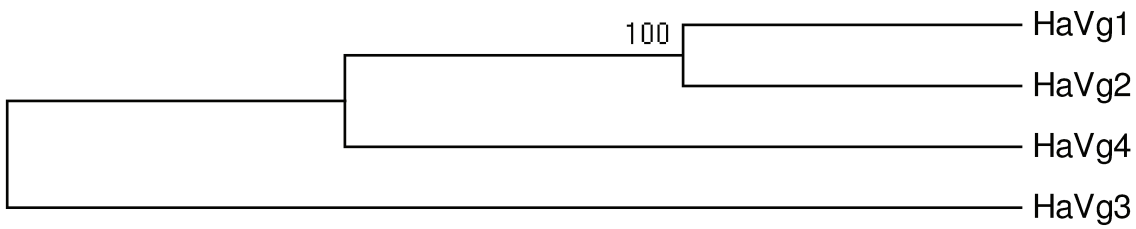

Figure 2.4: Unrooted phylogenetic neighbour-joining tree comparing American lobster (Homarus americanus) vitellogenin genes. Phylogenetic trees at a nucleotide and protein level were identical. Bootstrap value was set at 1000 replicates. Value at node represents bootstrap percentage for an unrooted tree with 4 taxa. The number of differences was used as the substitution model. A total of 566 positions was used in the final dataset. HaVg1: original vitellogenin sequence; HaVg2: EST similar to HaVg1 (85% at the nucleotide level and 74% at the protein level); HaVg3: EST similar to HaVg1 (55% at the nucleotide and protein level); HaVg4: EST similar to HaVg1 (65% at the nucleotide level and 44% at the protein level).

2.3.2 qPCR Results

From the microarray results, 15 genes of interest and 7 highly conserved genes were analysed. Of these primer sets, only 12 genes of interest and 3 highly conserved genes were deemed suitable for qPCR analysis (Table 2.1).

When compared to microarray results, RT-qPCR results showed more neutral expression levels across ovary stages (Figure 2.5). This was not the case however for the four vitellogenin genes. HaVg1 and 2 followed a similar expression pattern in both microarray and qPCR results, progressing from negative expression levels and increased to positive expression values. For qPCR data, all four vitellogenins progressed gradually from a downregulation in ovary stage 2 to an upregulation in ovary stage 5 (Figure 2.5; Panels I, J, K, L).

The highly conserved (reference) genes were the genes with the highest p-values indicating that they would, in theory, be the most stable genes to use for qPCR reference genes. This, however, was not the case, as qPCR analysis showed that genes were

variable (Figure 2.6). GeNorm analysis revealed that these genes had $p > 0.5$. With the low expression values of some of the genes of interest (Figure 2.5), all GOI were imported for GeNorm analysis to determine if suitable reference genes could be found. Inhibitor of growth protein 1-like, egg-derived tyrosine phosphatase, and growth-hormone inducible transmembrane protein were suitable (GeNorm $p < 0.5$) for use as reference genes. The remainder of qPCR analysis was done using these three reference unannotated genes.

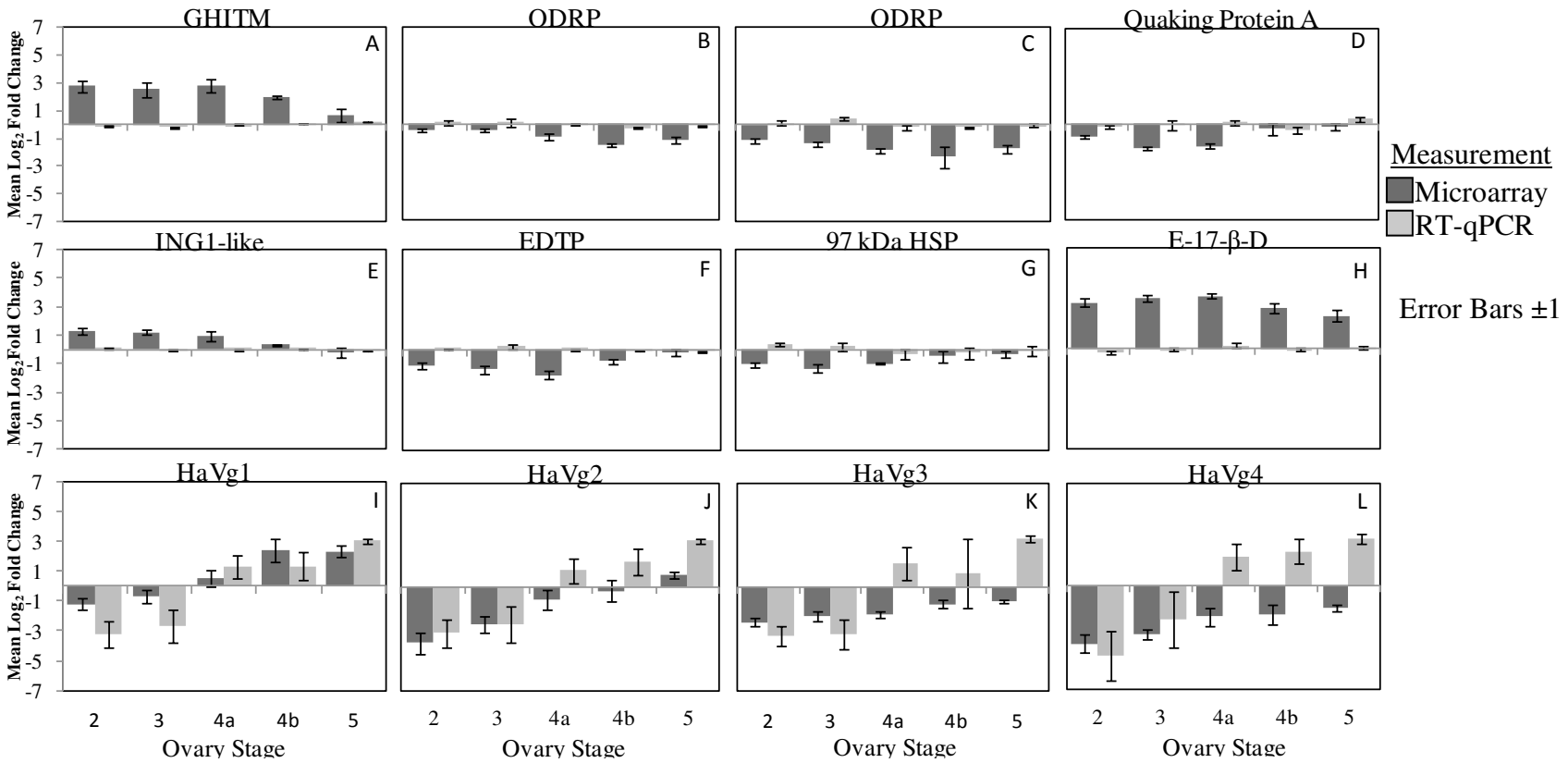

Figure 2.5: Comparison of inverse $\log_2$ (647/555) microarray data and $\log_2$ transformed RT-qPCR data. All data were normalized to average intensities. Significance was determined using ANOVA ($P < 0.05$). All microarray targets were significantly different. Bonferroni post-hoc tests were employed on RT-qPCR measurements. Significant differences between stages in RT-qPCR data are represented using different letters. Error bars were ± 1 standard error (SE). Panels represented individual genes. Genes were as follows; A: GHITM - Growth-hormone inducible transmembrane protein, B: ODRP – Ovary development-related protein; C: ODRP – Ovary development-related protein; D: Quaking protein A; E: ING1-like - Inhibitor of growth protein 1 like; F: EDTP - Egg-derived tyrosine phosphatase; G: 97 kDa HSP - 97 kDa Heat Shock Protein (egg-sperm receptor); H: E-17-β-D – Estradiol-17-β-dehydrogenase 12-B-like I: Vitellogenin HaVg1; J: Vitellogenin HaVg2; K: Vitellogenin HaVg3; L: Vitellogenin HaVg4.

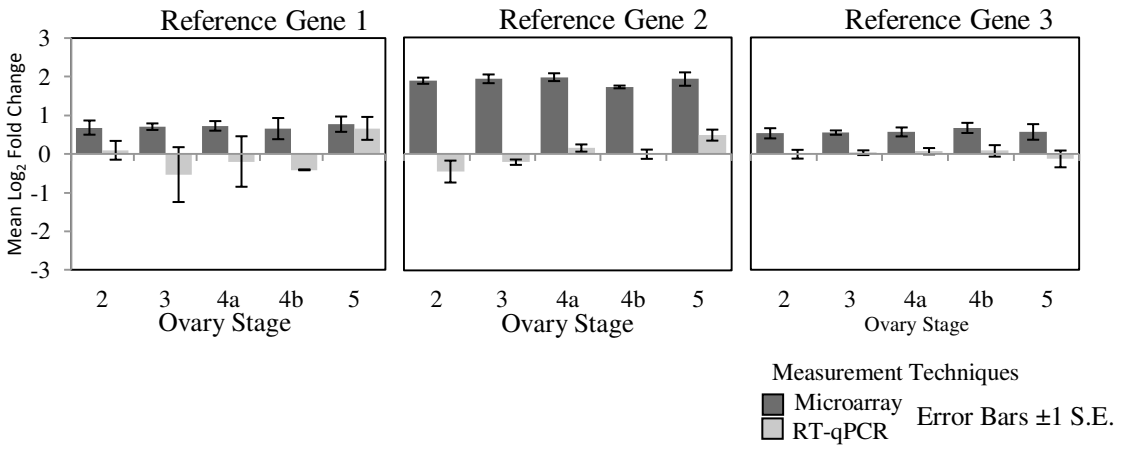

Figure 2.6: Comparison of expression for highly conserved genes between microarrays and RT-qPCR. Inverse $\log_2$ (647/555) microarray data plotted with $\log_2$ transformed RT-qPCR data. All data were normalized to average intensities. Significance was determined using ANOVA ($P < 0.05$). Microarray data was not statistically significant. Error bars are ± 1 standard error (SE).

2.4 Discussion

Ovary development in female American lobster progresses from ovary stage 1 until they extrude their eggs onto their pleopods at stage 6 when they are mature (Aiken and Waddy 1980). Stages 1 and 2 are usually white to yellow in colour. As vitellin, the yolk protein, accumulates in the oocyte, ovary colour will darken to a green. Females that have previously spawned, however, can have residual oocyte spots which show as yellow spots on the ovary (Comeau and Savoie 2002). Once oocytes have been extruded, ovaries will revert back to stage 3 (developing), which is a light green colour, but never back to stage 1 or 2 as ovaries are continually developing and are no longer immature (stage 1) or in early development (stage 2). Ovary stage 4 is subsequently split into stages 4a (immature) and 4b (mature) (Aiken and Waddy 1980). Current methods for staging female ovaries can be difficult at this stage, especially for females who follow a one-year reproductive cycle. This is because methods were created based on a typical two-year cycle and use season as a determining factor (Waddy and Aiken 2005). Female American lobsters can delay further ovary development at ovary stage 4a in response to environmental factors such as photoperiod, water temperature, and degree-days (Talbot and Helluy 1995; Waddy et al. 1995). Hence, because of the ability to delay ovary development up until and including ovary stage 4a and the inability to delay ovary development after the switch to ovary stage 4b, it was hypothesized that there would be a significant change in gene expression during the transition from stage 4a to stage 4b. Of the 7,716 genes which passed stringency tests, 1,774 genes were statistically significant. Of these 1,774 significant genes, 569 statistically significant genes (21.9%) were annotated.

2.4.1 Inter-stage Differences

Many of the genes that were functionally annotated showed inter-stage differences. The statistically significant functionally annotated genes were then assessed based on previously determined functions relating primarily to reproduction and growth. First, reproduction related gene functions were chosen because these genes would be involved in maturation and have the highest probability of differential inter-stage gene expression. Growth related functions, including moulting, were chosen as important factors due to the antagonistic roles they play with maturation (Aiken and Waddy 1976; Subramoniam 2000). As female American lobsters progress to ovary stage 4b, they will not moult before extruding and hatching their eggs (Comeau and Savoie 2002; Tsukimura et al. 2002; Waddy and Aiken 2005). Therefore, selecting genes based on this function seemed likely to reveal differential stage expression especially as it relates to reproductive commitment at ovary stages 4a and 4b. One of the major organs for controlling this balancing act is the x-organ, located in the eyestalk of many crustaceans including the American lobster (de Kleijn et al. 1998; Chung and Webster 2003; Nagaraju 2011).

Genes associated with growth and development are as follows: quaking protein A, inhibitor of growth protein 1-like (ING1), growth-hormone inducible transmembrane protein, and estradiol 17-β dehydrogenase. Although few were chosen, these were thought to be interesting due to their functional annotation being associated with growth along with their expression patterns.

Quaking protein A is involved in the development of blood vessels and the nervous system in mouse (Mus musculus) embryos. It aids in the development of. In the African clawed frog (Xenopus sp.) quaking protein A is vital in the early development of nervous

system and notochord development (Zorn and Krieg 1997). Quaking protein A is very similar to Wings-held-out protein in Drosophila sp. where it is involved in muscle development (Fyrberg et al. 1998). In female American lobsters, quaking protein A gene was downregulated in ovary stages 2 through 4a before becoming neutral in stages 4b and 5 in microarray results. qPCR results however were relatively neutral except downregulation equal to microarray expression in stage 4b (Figure 2.5; D).

Inhibitor of growth protein 1 (ING1) is involved in the suppression of tumour cells in humans, namely females (Garkavtsev et al. 1996; Campos et al. 2004). It is expressed in the yolk-sac of mice early in development before being transferred into developing bones (Zeremski et al. 1999). Although there are no published papers on the function of ING1 in crustaceans, it is highly conserved from humans through to the fruit fly (Drosophila melanogaster). Function in tumours range from cell cycle regulation to tumor growth (Campos et al. 2004; Aguissa-Touré et al. 2011). The negative regulation of cell growth was quickly evident in early studies of ING1 (Campos et al. 2004). The microarray data showed that there was an upregulation at stages 2 to 4a and a neutral expression at stages 4b and 5. RT-qPCR data was relatively neutral expression across all ovary stages (Figure 2.5; E).

Growth-hormone inducible transmembrane protein (GHITM) knowledge is limited to mainly human and mouse experiments. This gene is involved in the apoptotic release of cytochrome c, which is involved in the breakdown of glucose into energy (Goodsell 2004). Moulting consumes large amounts of energy (Saravanan et al. 2008). In Drosophila, GHITM mRNA was activated by aging flies (Zou et al. 2000; Yoshida et al. 2006). GHITM in combination with prothoracic gland-derived receptor occurs in

moulting insects. Thus, it was hypothesized that this gene would be upregulated at ovary stages 2 through 4a due to the increased probability of moulting pre-reproductive commitment. From microarray analysis, a constant upregulation from stages 2 to 4a was observed, before decreasing in expression at stage 4b and decreasing even more at stage 5. RT-qPCR data did not show the expected expression profile, instead there was neutral expression across all ovary stages (Figure 2.5; A).

Ovary Development-Related Proteins (ODRPs) were chosen because of their apparent function in ovary development in crabs and prawn (Ma et al. 2002; Jung et al. 2011). In the giant freshwater prawn (Macrobrachium rosenbergii), ODRPs were derived specifically from ovary tissues, implying function in the ovary although no other studies have investigated this gene or its function (Jung et al. 2011). The microarray expression profiles of these genes progressed from relatively neutral gene expression at ovary stage 2 decreasing to a maximum downregulation at ovary stage 5. However, this was not the case in the RT-qPCR data, where the expression profile remained neutral for all ovary stages (Figure 2.5; B, C). Both of these genes were $p > 0.05$ when using the Benjamini-Hochberg adjusted p-values. These genes did, however, fall within the non-adjusted $p \leq 0.05$ cut-off. This could partially explain the inconsistency as the case of false positives increases above the adjusted statistical significance p-value.

Egg-Derived Tyrosine Phosphatase (EDTP) is involved in oogenesis and embryogenesis in a variety of invertebrate species including the fruit fly, Drosophila melanogaster, the flesh fly, Sarcophaga peregrina, and the European honey bee, Apis mellifera (Yamaguchi et al. 1999; Yamaguchi et al. 2005; Cardoen et al. 2011). According to Yamaguchi et al. (2005), EDTP is essential in early ovarian development in the fruit fly. During early

embryogenesis, EDTP is found in significant amounts in egg yolk but rapidly deteriorates as it nears larval stages. Although the complete roles that EDTP plays in Drosophila development has not been determined, Yamaguchi et al. (2005) hypothesized that this gene may be involved in process such as nuclear division, nuclear migration, or as a component of a signal transduction pathway. Contrary to results from previous studies (Yamaguchi et al. 2005), our microarray data showed downregulation to stage 4a prior to becoming relatively neutral at stage 5. RT-qPCR showed poor expression where there was neutral expression across all ovary stages (Figure 2.5; F). As with ODRP, the significance falls outside the cut-off when the p-value is adjusted but within the significance range of the non-adjusted p-value.

The 97 kDa Heat Shock Protein (HSP) in purple sea urchins (Strongylocentrotus purpuratus) is expressed on the egg surface just prior to fertilization, facilitating the binding of sperm to egg (Mauk et al. 1997). Because of this need, expression should have been upregulated as ovaries approach and reach stage 6 prior to oocytes being extruded and fertilized. Our microarray results depicted a downregulation at ovary stage 2 progressing to a relatively neutral expression at stage 5. This follows a general positive linear pattern. This however was not in accordance with RT-qPCR results which varied between up and downregulation. These variations were all centered around neutral expression (Figure 2.5; G). This discordance could be caused, again, by the failure to meet the Benjamini-Hochberg correction's statistical significance cut-off of a p-value of 0.05. This gene was, however, statistically significant when the Benjamini-Hochberg correction was removed.

Estradiol 17-β-dehydrogenase 12-B-like is used in the balancing of estrone and estradiol in the system. In higher vertebrates, estrone is involved in vitellogenin synthesis and oocyte maturation. In the freshwater prawn (Macrobrachium rosenbergii), estradiol 17-β peaks in expression in both ovary tissue and hemolymph as oocytes are maturing before steadily decreasing as they become gravid (Ghosh and Ray 1993). Due to the apparent control of this gene in the prawn, expression should have been upregulated in early ovary stages for the American lobster and downregulated in the latter stages. In the microarray data, the Estradiol 17-β-dehydrogenase 12-B-like gene expression was relatively constant at a high upregulation before decreasing to a weaker upregulation in stages 4b and 5. Again, RT-qPCR data were constant in all ovary stages, with expression remaining neutral in all ovary stages (Figure 2.5; H).

Lastly, four vitellogenin genes (HaVg1-4) were statistically significant in both microarray and RT-qPCR results . Vitellogenin is a precursor to vitellin which is an egg yolk protein (Tsukimura et al. 2002; Tiu et al. 2009). Vitellogenin is present in hepatopancreas and the ovary, although the four Homarus vitellogenins have not been categorized as either hepatopancreas or ovary derived. The vitellogenin is transported from the hepatopancreas, where it is synthesized, to the ovary through the hemolymph (Tsukimura et al. 2002; Tiu et al. 2009). Tiu et al. (2009) previously identified the Homarus americanus vitellogenin gene HaVg1. They have, however, confirmed the possibility of multiple vitellogenin genes in the American lobster. When comparing the 80 kDa protein sequence derived from American lobster ovary and hepatopancreas, there were differences (Tiu et al. 2009). The ovary and hepatopancreas may express two different vitellogenins and they may be stage dependant as in other crustaceans (Tsang et al. 2003;

Mak et al. 2005; Tiu et al. 2009). Tiu et al. (2009) also confirmed the similarity of vitellogenin genes isolated from other crustaceans to HaVg1. The vitellogenin from the crayfish, Cherax, is 57% similar to HaVg1 at a protein level whereas vitellogenin from several prawn, Metapenaeus species, were between 40-43% identical to HaVg1 (Tiu et al. 2009).

Four vitellogenin or vitellogenin-like genes were found within the EST sequence database. The complete Homarus americanus vitellogenin gene, HaVg1 (GenBank accession number: EF422415.1) was statistically significant between stages. Another EST (GenBank accession number: EX568231.1) was 74% similar to HaVg1 at the protein level and was given the abbreviation HaVg2. A third EST (GenBank accession number: EW997929.1) showed the most similarity to Cherax quadricarinatus (GenBank accession number: AAG17936.1) vitellogenin. This gene was 55% similar to HaVg1 at the protein level and was named HaVg3. The fourth EST (GenBank accession number: FD467667.1) was most similar to the Metapenaeus ensis vitellogenin (GenBank accession number: AAM48287.1) and shared 44% similarity to HaVg1 and was named HaVg4.

Vitellogenin levels in American lobster hemolymph progress from low levels during early maturity prior to increasing to maximum expression levels in mature oocytes (Tsukimura et al. 2002; Tiu et al. 2009). Regardless of sequence variation, all four vitellogenins followed a similar expression profile. The total vitellogenin expression of all 4 vitellogenins progressed from a downregulation at stage 2 to an upregulation at stage 5 (Figure 2.5; I, J, K, L).

Several other primer sets for GOI were chosen for RT-qPCR analysis but were not able to be optimized. Some of these failures were surprising as they had quite significant microarray results both in the corrected and non-corrected p-values. One of these genes was Egghead (Egh) CG9659-PC, isoform C, of Drosophila melanogaster. The Egh group of genes are involved in oogenesis (Fan et al. 2005). After Benjamini-Hochberg correction, the Egh gene had $p = 0.01433$ but the primers were not able to be optimized because they contained multiple bands at multiple annealing temperatures. These bands could be due to primer dimers, although every precaution was taken to avoid them. Primer dimers appear when forward and reverse primer bind and amplify creating a second band when run on an electrophoresis gel. Primer dimers are detrimental to RT-qPCR results as they obscure the true quantitative values (Vandesompele et al. 2002). In future analyses, these should be run with new primer sequences.

2.5 Research Obstacles

These results are representative of total gene expression in four tissues (hepatopancreas, ovary, eyestalk, and haemocyte pellet). These four tissues were chosen based on apparent reproductive function and pooling was done as a cost saving measure during the first analysis of gene expression in an attempt to delineate new biomarkers for evaluating reproductive status of female American lobsters. By combining these four tissues, the ability to accurately examine how genetic expression differentiates from one tissue to another and how they are expressed in each tissue at each individual ovary stage is lost. The combination of these four tissues may also dilute gene expression profiles. For example, if 1 gene was upregulated in 1 tissue and not in the other 3 tissues from the same lobster, the upregulation of that gene would end up being less than what it truly is.

These tissues were combined to get preliminary results of gene expression relating to ovary development in American lobsters using microarray technology. Examination of these four tissues individually would require quadruple the number of microarrays, reagents, time and ultimately cost. One primary purpose of this study was to determine potential biomarker genes, with the hope of one or more being from non-lethal sampling sites such as haemocyte pellets or eyestalk. One major problem encountered was the low levels of RNA available from resuspended haemocyte pellets. When adding Tri-Reagent to the solid haemocyte pellet, the tip of the pipet needed to be used to break apart the solid pellet prior to centrifugation to obtain maximum amounts of RNA. Total RNA levels from eyestalk samples were also low compared to that of hepatopancreas and ovary which contained high levels of RNA.

American lobsters with an ovary stage at 4b were scarcely represented biologically. This may have been partly due to the time of year that the American lobsters were collected. According to Waddy and Aiken (2005), the ideal time for collecting females with the highest chance of being at stage 4b is in the spring, around May. It is also quite difficult to obtain an American lobster at ovary stage 4b because once the ovary progresses to this stage it will rapidly progress to ovary stage 5. One way to combat this lack of stage 4b lobsters is to collect more American lobsters earlier in the fishing season. This will increase the probability of collecting stage 4b American lobsters. This will also increase biological samples in all other ovary stages.

A major problem encountered in this study is that the American lobster genome is not fully annotated. Only 32% of significant genes were functionally annotated. This means that 68% of statistically significant genes were not examined due to lack of functional

annotation. In this 68%, there may be a gene which better represents reproductive commitment and may have the possibility of finding non-lethal ovary staging method.

Female American lobsters were collected in the summer of 2009 and 2010. Ideally, two-way ANOVA would be used to determine the effect of year on each gene. This could not be done because certain ovary stages were not present in both year classes.

2.6 Conclusion

This was the first assessment of reproductive status of female American lobster using microarray technology by evaluating gene expression profiles from ovary stages 2 through 5. Of the genes which passed flagging parameters, only ~23% of genes were statistically significant. Only 32% of statistically significant genes were functionally annotated.

Molecular and cellular processes dominated the five clusters. Genes associated with reproduction, although present in all clusters, represented a maximum of 10 genes/cluster. Growth related genes were found in all but cluster 5. The four clusters where growth was present, there was a maximum of 3 genes in each cluster. From these functional annotations, genes were chosen for RT-qPCR analysis. The most consistent microarray and RT-qPCR results were the four vitellogenin genes. The progression from downregulated gene expression early in ovary stages to upregulation in the later stages was consistent with literature for American lobster (Tsukimura et al. 2002) and other crustaceans, such as the banana shrimp, Fenneropenaeus merguiensis, green crab, Carcinus maenus, and Kuruma prawn, Penaeus japonicus (Tsutsui et al. 2000; Phiriyangkul et al. 2007; Ding et al. 2010). In the Kuruma prawn, the expression of mRNA in vitellogenin differed between ovary and hepatopancreas vitellogenins; they

increased to either stage II or III, respectively (Tsutsui et al. 2000). In the green crab, vitellogenin levels increased from low expression in pre-vitellogenic stages to peak at stage 2 vitellogenic ovaries in the ovary, although relative expression values were low. Hepatopancreas levels increased significantly from previtellogenic stages but remained stagnant across the 3 vitellogenic stages of the ovary (Ding et al. 2010). The banana shrimp peaked in expression at stage 2 ovaries for ovaries and steadily increased to stage 4 ovaries in the hepatopancreas (Phiriyangkul et al. 2007). This also shows the difference in vitellogenin expression between ovaries and hepatopancreas.

With lack of concurrence between microarray results and RT-qPCR, results should only be used with a $p < 0.05$ after Benjamini-Hochberg correction had been performed. This was applied to reduce false discovery rates (FDR) (Quackenbush 2005; Tabangin et al. 2007). Within the results, a dramatic limitation was observed for significantly different expression and therefore data were assessed using traditional, non-corrected p-values to discuss trends.

This experiment combined four tissues for each gene. Genes are, however, regulated on an individual tissue level. This pooling of tissues will give a total gene expression for the combined tissues but individual tissue regulation of genes may be missed. For example, although vitellogenin increases from downregulation at ovary stage 2 and increases to upregulation at ovary stage 5 in the ovary does not mean that the expression profile is the same in the hepatopancreas. This tissue specific expression profile is seen in the Kuruma prawn where the highest expression level of vitellogenin in the ovary is in the early exogenous vitellogenic stage (II) and decreases to late exogenous vitellogenic stage (IV) but the hepatopancreas vitellogenin levels increase to late exogenous vitellogenic stage

(III) before decreasing to late exogenous vitellogenic stage (IV) (Tsutsui et al. 2000). In future research, the microarray experiments should be conducted on individual tissues to obtain in-depth expression profiles for each organ thought to play a role in ovary development rather than pooling RNA from different tissue samples.

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CHAPTER 3: GENERAL DISCUSSION

3.1 Summary of Findings

Investigation of reproduction and endocrinological control in the American lobster, Homarus americanus has been the topic of several studies (Soyez et al. 1987; Nelson et al. 1988; Tensen et al. 1989; Tsukimura and Borst. 1992; de Kleijn et al. 1998; Tsukimura et al. 2002; Tiu et al. 2009; Tiu et al. 2010). The present study was the first to use microarray technology to examine female reproduction in the American lobster. After flagging had taken place there were only 7,716 genes which remained. Of these, only ~37% of genes were functionally annotated. Genes which were statistically significant only represented ~23% with only about 32% of those being functionally annotated.

Within these functionally annotated genes that showed significant differences in gene expression were genes coding for reproduction, growth, and development although the majority of genes coded for cellular and metabolic processes (Appendix S). Genes selected for further study by complementary RT-qPCR were chosen from the functionally annotated genes to validate microarray results. Four specific Homarus americanus vitellogenin genes were identified that showed consistent gene expression among microarrays and RT-qPCR. Three new vitellogenin genes were identified based on sequence similarity at protein and nucleotide sequence level as compared to HaVg1 and other crustacean vitellogenin sequences within GenBank. These new vitellogenin ESTs were labelled HaVg2 (85% similar at a nucleotide level and 74% similar at a protein level to HaVg1), HaVg3 (55% similarity at both the nucleotide and protein levels to HaVg1) and HaVg4 (65% similar at a nucleotide level and 44% similar at a protein level to HaVg1). Multiple vitellogenin genes have been identified in other crustacean species and

were hypothesized to be in the American lobster (Tsang et al. 2003; Kung et al. 2004; Mak et al. 2005; Tiu et al. 2009).

The expression of these four genes progressed from being downregulated in early ovary stages (2 and 3) before being upregulated in ovary stages (4b and 5). This mRNA expression pattern agreed with previous data from the American lobster and other crustacean species based on both gene and protein expression (Tiu et al. 2008; Tiu et al. 2009; Ding et al. 2010; Ferre et al. 2012). Although these results are expressed as total gene expression across 4 tissues (hepatopancreas, ovary, eyestalk and haemocyte pellet), it is probable that each of the vitellogenins are expressed differently in each individual tissue. This has been observed with many other crustaceans where expression differs between hepatopancreas and ovaries (Tsutsui et al. 2000; Phiriyangkul et al. 2007; Ding et al. 2010; Ferre et al. 2012).

3.2 Future Directions

Although the current study provided a good framework for the first American lobster reproductive microarray experiment, there were several limitations. Future studies should examine the following to enhance the knowledge of reproductive female American lobsters:

- Examining gene expression from individual tissues. Examination of pooled tissues, although cost effective, was limited in discerning individual tissue expression patterns that may be more diagnostic due to the implications of dilution effects.

- Increasing the number of biological samples, especially at ovary stages 4a and 4b. There was a small number (n=3) of American lobsters with ovaries at stage 4b. Increasing the numbers at this stage would, in theory, decrease the variability in expression and may provide a more thorough assessment of gene expression profiles.
- Having all samples collected the same year, or ensuring that the biological samples capture all ovary stages to allow for two-way ANOVA statistics for year to year comparisons. The current results were constrained to solely one-way ANOVA due to certain samples lacking in 1 year.
- The examination of hemolymph as a possible non-lethal test for ovary development and reproductive commitment. Hemolymph transports proteins, such as vitellogenin, and could contain valuable information on ovary development. This could be explored with other methods including proteomics.
- Obtaining the full American lobster DNA sequence for HaVg2, HaVg3, and HaVg4. These genes were classified based on ESTs alone. HaVg2, HaVg3 and HaVg4 contain 609, 739, and 592 nucleotide base pairs, respectively. In comparison to HaVg1, which contains 8,518 nucleotide base pairs, there is a definite gap in knowledge and having complete sequences could provide more meaningful information about tissue specificity for future expression analyses.
- Further progress in functionally annotating the American lobster genome will increase the understanding of specific genes. A significant amount of data was 'lost' due to the lack of functional annotation within the available genome data. This is a problem for all non-model organisms; further research in functional

assessment of genes and annotations may lead to significant findings about reproductive commitment.

Due to the continuing lack of knowledge pertaining to reproductive commitment in American lobsters, the aforementioned points should be examined extensively. The commercial importance of this species and the lethality of the current ovary staging methods employed highlight the need and importance of continued research. Results from the present study could be used to aid researchers in the quest for genes resulting in reproductive commitment and ovary maturation. Studies focused on individual tissues with large numbers of female American lobsters would increase the probability of finding the genetic marker(s) responsible to reproductive commitment in Homarus americanus.

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Appendix A - Lobster Necropsy and Tissue Homogenization SOP

Modified from Fraser Clark (AVCLSC). 2010.

Before the necropsy begins on each animal:
Necropsy area should be cleaned and a fresh bench coat applied
All molecular biology necropsy tools are clean (forceps and scissors)
Arrange balance and weigh boats appropriately
OMNI Homogenizer has been cleaned and reassembled

1. Perform physical assessment of each female lobster
2. Remove 0.5 mL of hemolymph into 4.5 mL of Artificial Seawater (ASW) with formalin for haemocyte counts
3. In a new syringe, draw 2-3 mL of hemolymph. Place 1 drop in 2x Tryptic soy broth (TSB), 2x Phenyl Ethyl Alcohol (PEA), and 2x ciliate media
4. Using another syringe, draw 10 mL of hemolymph and add to 15 mL tube- spin on Beckman T-J centrifuge (level 7 for 5 min)
5. Remove the plasma (supernatant) and keep the haemocyte pellet
6. Euthanize the animal by severing the ventral nerve cord anterior to the first periopods
7. Once defensive posture and eyestalk withdrawal cease, remove the carapace by making an incision along the dorsal midline of the thorax and then 45° to the left and right of the head
8. Remove the appropriate amount of tissue from the lobster (see below), rinse thoroughly with sterile filtered ASW and place the tissue in 15 mL tube containing Tri Reagent

Amount	Tissue
1000 mg	Ovary Hepatopancreas
500 mg	Eye/eyestalk
Pellet of 10 mL	Haemocyte pellet

9. Homogenize the tissue using the Omni Tissue Homogenizer until the Tri Reagent/tissue homogenate is homogenous
10. The tissue homogenizer must be rinsed three times with sterile ASW, and once with Tri Reagent between each tissue
11. Snap freeze all 15 mL tubes with tissues after all of the tissues have been homogenized and incubated in Tri Reagent at room temperature (23°C) for at least 5 min
12. Place snap frozen 15 mL tubes in a storage box and place in the -80°C freezer

Appendix B – Tri Reagent SOP

Created by Fraser Clark (AVCLSC). 2010.

The Tri Reagent make-up was modified from Chomczynski and Sacchi (1987) by Fraser Clark of the AVCLSC. Tri Reagent is a cheaper substitute for commercially available TRIzol (Invitrogen, Carlsbad CA). The original Tri Reagent make-up was modified and sold as TRIzol. When handled appropriately, Tri Reagent is as effective for the extraction and purification of RNA as TRIzol.

Tri Reagent		Conc
Guanidine Isocyanate (Biotechnology Grade) (BioShop # GUA004.500)	16.54 g	1.4M
Phenol (Biotechnology Grade) Saturated pH 4.5 (BioShop # PHE511.400)	38 mL	38%
Glycerol (Sterile) (BioShop GLY003.500)	5 mL	5%
Sodium Acetate (BioShop SAA 310.500)	0.8203 g	0.1M
diH2O (start with 25 mL)	up to100 mL	

In a clean 1L bottle, add ~25 mL of diH_2O followed by guanidine isocyanate, sodium acetate, and glycerol. Mix via stir bar on benchtop. Place bottle in the fume hood and add phenol, as phenol produces noxious gas. Add the remaining diH_2O while under the fume hood. Make up Tri Reagent no more that 24h before you intend to use it, and store it in the fridge at 4°C. Be sure to properly label Tri Reagent with the appropriate warnings.

Chomczynski, P., Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry, 162: 156-159.

Appendix C – Reproductive Status RNeasy® Mini RNA Extraction SOP

Modified from Fraser Clark (AVCLSC). 2010.

1. Remove ovary, eye, hepatopancreas, and haemocyte pellet from -80°C freezer. Thaw at room temperature (23°C).

2. Add 200 µL of chloroform for every 1 mL of homogenate in Tri Reagent and shake well to mix. Allow the mixture to sit at room temperature (23°C) for 3 min.

3. Using the tip of a pipet, mix the haemocyte pellet with the chloroform to resuspend/release the solid pellet.

4. Pre-chill A-10-250 rotor to 4°C and centrifuge for 15 min @ 12,000 x g @ 4°C with the Allegra 25R centrifuge.

5. In the fume hood, carefully remove 600 µL of the top aqueous phase and place it into a clean 1.5 mL tube.

6. Slowly add an equal volume (600 µL) of 70% EtOH (Molecular Biology Grade) to the aqueous phase and mix via pipetting.

<u>The following steps require a Qiagen RNeasy Kit</u>

7. Proceed to Step 6 of the RNeasy® Mini handbook

   Add 600 µL the aqueous phase/EtOH solution to a labelled spin column
   Centrifuge at 8,000 x g for 30s at 23°C
   Discard the flow through
   Add the remaining 600 µL of solution to a labelled spin column
   Centrifuge at 8,000 x g for 30s at 23°C
   Discard the flow through

8. Add 350 µL of RW1 buffer to the column
   Centrifuge at 8,000 x g for 30s at 23°C
   Discard the flow through

9. Take a DNase Treatment I (Qiagen, Valencia CA) aliquot out of the freezer
   You need 10 µL of DNaseI and 70 µL of Buffer RDD for every column
   Mix only by inversion- DNaseI is very sensitive to physical degradation

   Note: DNase is shipped freeze dried and must be rehydrated prior to use with 550 µL of RNase free H_2O. This is then aliquoted into 10 x 55 µL aliquots and stored at -20°C.

10. Add 80 µL of DNaseI reaction buffer to column
    Incubate at room temperature (23°C) for 15 min

Centrifuge at 8,000 x g for 30s at 23°C
Discard flow-though

11. Add 350 µL of RW1 buffer to the column
Centrifuge at 8,000 x g for 30s at 23°C
Discard flow-though

12. Add 500 µL of RPE buffer to the column
Centrifuge at 8,000 x g for 30s at 23°C
Discard flow-though

13. Add 500 µL of RPE buffer to the column
Centrifuge at 8,000 x g for 30s at 23°C
Discard flow-though
Centrifuge again at 8,000 x g for 30s at 23°C

14. Place the column in a new 1.5 mL tube and place 30 µL of nuclease free H_2O onto the column. Let column sit for ~1 min and then centrifuge @ 6,000 x g for 2 min. Repeat with another 30 µL of nuclease free H_2O into the same tube.

15. Aliquot 2 µL of sample into a sterile 0.5 mL micro centrifuge tube for NanoDrop quantification and add 8 µL of RNase free H_2O. Aliquot the sample out 3 times in 2 µL volumes into another 0.5 mL tube, analyze RNA quality with the Bio-Rad Experion™. The remaining RNA should be stored immediately at – 20°C.

Appendix D – NanoDrop Analysis

Modified from NanoDrop (Thermo Scientific, Waltham, MA) method. 2010.

1. Remove aliquot from -80°C freezer and allow to thaw at room temperature (23°C).
2. Pipet 1.5 µL of Nuclease-free H_2O onto the pedestal of the NanoDrop prior to initiating the device. Once measurement is complete, wipe the surface with a Kimwipe®.
3. Dispense another 1.5 µL nuclease-free H_2O onto pedestal, choose the proper nucleic acid type (RNA, DNA, ssDNA), and blank the measurement. Wipe with a Kimwipe® when complete.
4. Pipet 1.5 µL of sample onto the pedestal to measure the absorbance at 260 and 280 nm. Wipe the sample off with a new Kimwipe®.
5. Measure each sample in triplicate to obtain an average reading.
6. After 9 samples have been measured, measure 1.5 µL of nuclease-free H_2O to ensure no residual sample.
7. Once all measurement are completed, examine the nucleic acid concentration and the 260/280 nm ratio to determine nucleic acid quantity and ensure purity of the sample.

Note: 260 nm is the absorbance wavelength of nucleic acids while 280 nm is that of proteins and o*ther contaminants. A pure sample should have a 260/280 ratio of ≥ 1.9.*

Appendix E – Bio-Rad Experion™ RNA StdSens Analysis SOP

Created by Fraser Clark (AVCLSC). 2010.

Note: *New kits of RNA ladder should be aliquoted in 1 µL volumes in 0.5 mL* tubes to avoid freeze/thaw degradation.

1. Acclimatize the tubes of filtered gel, RNA gel stain and loading buffer to room for ~15 min. Remove the sample from the -80°C freezer and allow to thaw. The RNA ladder should be kept on ice at all times. Before using any reagent, briefly vortex and centrifuge it. **CAUTION:** Protect the gel stain from light at all times.

2. Clean the Experion™ electrodes by filling the cleaning chip with 800 µL of electrode cleaner and place into the Experion, close lid and wait two min before removing the chip. Remove the cleaning solution from the chip and replace with 800 µL of DEPC water to rinse the electrodes. Place the chip into the Experion™ and then remove after 5 min and discard the water. Leave the Experion™ lid open for ~1 min to allow electrodes to dry.

3. When using a kit for the first time, the RNA gel must be filtered. Place 600 µL of RNA gel into a supplied spin filter. Centrifuge gel at 1,500 x g for 10 min and remove the flow-through (filtered gel). Place it into a clean 1.5 mL tube.

4. Take 65 µL of filtered gel and add 1 µL of RNA stain and vortex to make the Gel-Stain solution (GS). Cover this with tinfoil to protect from light.

   Note: Unused filtered gel may be stored in the fridge (4°C) for up to one month.

5. Add 18 µL of nuclease-free H_2O to each 2 µL aliquot to dilute 10-fold.

6. Place 2 µL of each sample into clean RNase-free 0.5 mL microcentrifuge tubes and place 1 µL of RNA ladder into another 0.5 mL microcentrifuge tube.

7. Denature samples and ladder by placing onto thermoblock for 3 min at 70°C. Briefly spin down the tubes and place on ice for 5 min.

8. Remove a RNA StdSens chip from its package and place 9 µL of GS solution into gel priming well labelled with a highlighted "GS" on the chip. Place chip into priming station, close lid and set Pressure to **B**, time to **1** and press the **Start** button. Remove after priming and inspect the microchannels on the underside of the chip for any bubbles without turning over. If bubbles can be seen, simply tap the chip right side up on the bench top to remove them.

9. Pipet 9 µL of GS solution into the other well labelled "**GS"** and 9 µL of filtered gel into the well labelled "**G"**.

10. Pipet 5 µL of loading buffer into tubes containing samples and ladder. Mix with pipet.

11. Add 5 µL of denatured RNA ladder/loading buffer into the well labelled "**L**" and 5 µL of denatured sample/ loading buffer into the twelve sample wells.

    Note: *Unused wells should have 5 µL of loading buffer in them.*

12. Within 5 min of loading the chip, place it into the Experion™. Ensure the Experion™ is turned on and then launch the software. Select "**New Run**" and choose the assay that you want to run (i.e.: Eukaryotic or Prokaryotic Total RNA or Eukaryotic mRNA) and click on the "**Start"** button. Once the run is complete, remove and discard chip.

13. Clean the Experion™ electrodes by placing 800 µL of DEPC water into the cleaning chip and placing it into the Experion™ for 1 min then remove the chip and leave the Experion™ lid open for 1 min to dry.

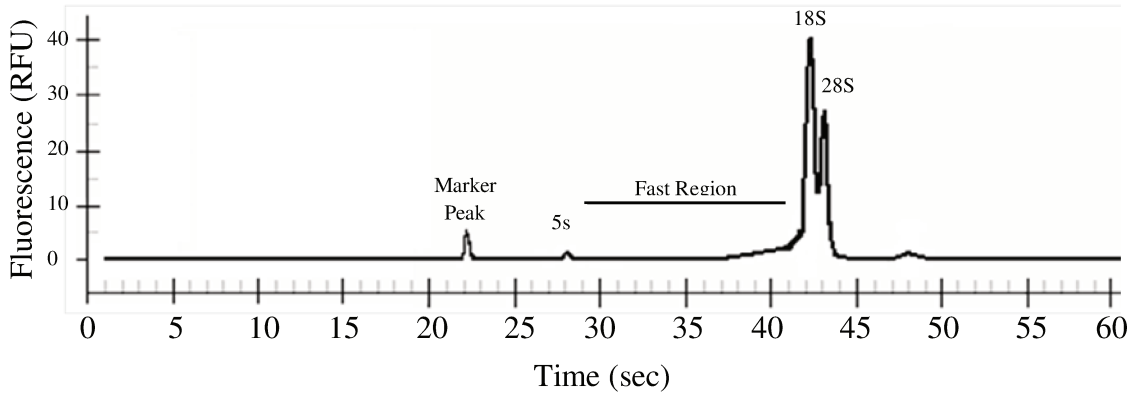

Figure A1: 10-fold dilution of RNA extracted from haemocyte pellet sample (Lobster F40). Clearly defined 18S and 28S regions and a homogenous fluorescence reading within the fast region are markers of high RNA quality. Fluorescence is measured in relative fluorescent units (RFU) and time in sec.

Appendix F - cDNA Synthesis SOP

Created by Fraser Clark (AVCLSC). 2010.

Reagent Kits Required:

Invitrogen SuperScript™ Plus Indirect cDNA Labelling System Cat # L1014-06

cDNA Synthesis

1. Prepare the following:
   - 20 µg of total RNA
     - 6 µg of ovary, hepatopancreas, and eye
     - 2 µg of haemocyte pellet
   - 2 µL of Anchored oligo(dT)20 Primer (2.5 µg/µL)
   - DEPC-treated H_2O to 18 µL

2. Mix, pulse and Incubate at 70°C for 5 min
   - Use thermocycler with heated lid
   - Place reactions on ice for at least 1 min

3. Add the following to each tube on ice:
   - 6 µL of 5X First strand buffer
   - 1.5 µL 0.1M DTT
   - 1.5 µL dNTP mix
   - 1.0 µL RNaseOUT
   - 2.0 µL SuperScriptIII RT
   - Mix solution and pulse briefly

4. Incubate solution at 46°C for 2-3 h
   - Use thermocycler with heated lid

Alkaline hydrolysis and Neutralization

1. Add 15 µL of 1 N NaOH to each reaction tube.
   - Mix and pulse briefly

2. Incubate tubes at 70°C for 10 min

3. Add 15 µL of 1 N HCl
   - Mix and pulse briefly

Purification of the First Strand

1. Add 700 µL of Binding buffer (with Isopropanol added) to a new 1.5 mL tube

2. Add neutralized cDNA to Binding buffer

Mix by flicking tube or inversion

3. Add binding buffer/ cDNA solution to a Low-Elution volume spin column
   Spin at 3,300 x g for 1 min at 23°C
   Discard the flow-through

4. Add 600 µL of wash buffer to the spin column
   Spin the column at 10,000 x g for 30s at 23°C
   Discard the flow-though
   Spin the column again at 10,000 x g for 30s at 23°C
   Place the column in a new amber collection tube

5. A 15 µL of DEPC-treated H_2O to the centre of the spin column
   Incubate at room temperature (23°C) for 1 min
   Spin at 10,000 x g for 1 min at 23°C
   Keep eluate
   Add an additional 15 µL of DEPC-treated H_2O to the spin column
   Incubate at room temperature (23°C) for 1 min
   Spin at 10,000 x g for 1 min at 23°C

6. Store the cDNA at -20^{o}C for up to 1 week
   At -80^{o}C for more than 1 week
   At 4^{o}C for immediate use

Appendix G – aRNA synthesis SOP

Created by Fraser Clark (AVCLSC). 2010.

Reverse Transcription (RT) for 1st strand cDNA synthesis

1. Make up RT-Master Mix

1 µL of Nuclease free H_2O	4 µL of dNTP Mix
1 µL of T7 Oligo(dT) Primer	1 µL of RNase Inhibitor
2 µL of 10x First Strand Buffer	1 µL of ArrayScript

---

20 µL (Add enough Total RNA (1-5 µg) and then enough Nuclease free water to make a 10 µL volume)

300 ng of ovary, hepatopancreas, and eye
100 ng of haemocyte pellet

Note: Mix RT-Master Mix via flicking and then pulse mix down

2. Incubate at 42°C for 2 hrs on Thermocycler (with heated lid) and then centrifuge tubes down to collect contents and place of ice.

Synthesis of 2nd strand cDNA

1. Make up 2nd Strand Master Mix on Ice

63 µL Nuclease-free Water	2 µL DNA Polymerase
10 µL 10X Second Strand Buffer	1 µL RNase H
4 µL dNTP Mix	

---

80 µL (Mix components by flicking and then centrifuge briefly to collect master mix)

Transfer the 80 µL of Second Strand Master Mix into 1st Strand reaction tube, mix by flicking the tube and centrifuge briefly again. Incubate reaction tube on thermocycler @ 16°C for 2 hrs (Note: Ensure that heated lid is turned **off** as temperatures above 16°C will compromise yield)

2. After incubation place tubes on ice.

cDNA Purification

1. Add 250 µL of cDNA binding buffer to 1.5 mL tube
Add 100 µL of 2nd Strand cDNA to 1.5 mL tube

Mix by flicking tube
Pulse

2. Add cDNA solution to cDNA filter cartridge
Spin @ 10,000 x g for 1 min at 23°C
Discard flow-through

Add 500 µL of wash buffer to cDNA filter cartridge
Spin @ 10,000 x g for 1 min at 23°C
Discard flow-through
Spin @ 10,000 x g for 1 min at 23°C
Place cDNA filter cartridge into a cDNA elution tube

3. Elute cDNA w/ 18 µL of 55°C Nuclease-free H_2O
Leave at room temperature (23°C) for 2 min
Spin at 10,000 x g for 1 min at 23°C

<u>In-vitro Transcription to Synthesize Amino-Allyl modified aRNA</u>

1. Make up IVT Master Mix
3 µL of aaUTP (50 mM)
12 L ATP, CTP, GTP mix
3 µL UTP Solution (50 mM)
4 µL T_7 10X Rxn buffer
4 µL T_7 Enzyme mix

Note: Mix via flicking and then pulse mix down

2. Add 26 µL IVT Master Mix to all cDNA
Mix gently
Pulse briefly

3. Incubate @ 37°C for 14 hrs

4. Stop reactions by adding 58 µL of nuclease-free water
Mix gently
Pulse briefly

<u>aRNA Purification</u>

1. Add 350 µL aRNA binding buffer to 1.5 mL tube
Add 100 µL of sample
Add 250 µL 100% EtOH

Note: Mix gently, but <u>DO</u> <u>NOT</u> centrifuge

2. Pipet mix to an aRNA filter cartridge/collection tube
Spin @ 10,000 x g for 1 min at 23°C

Discard flow-through

3. Add 650 µL of Wash Buffer
Spin @ 10,000 x g for 1 min at 23°C
Discard flow-through
Spin @ 10,000 x g for 1 min at 23°C

4. Elute with 200 µL Nuclease-free H_2O
Incubate on 55°C heat block for 10 min
Spin @ 10,000 x g for 1.5 min at 23°C
Store @ -20°C or NanoDrop

NanoDropping aRNA

1. Make a 1 in 5 dilution of the labelled aRNA for measuring on the NanoDrop
Mix tube well by flicking
NanoDrop 1.5 µL on "RNA 40" setting. Repeat 3 times
Aliquot in 6 µg amounts

Appendix H – cDNA labelling for microarray hybridization SOP

Created by Fraser Clark (AVCLSC). 2010.

Reagent Kits Required:

Invitrogen SuperScript™ Plus Indirect cDNA Labelling System Cat # L1014-06

cDNA Synthesis

1. Dry purified first strand cDNA synthesis to 0.5-3µL using the Genevac system
   Preheat to 51-54°C

2. Add 5 µL of 2X coupling buffer to concentrated cDNA

3. Add 2 µL of DMSO (room temperature (23°C) for 10 min) to an AlexaFluor® reactive dye
   Vortex lightly and pulse down

4. Add DMSO/Dye solution to cDNA
   Vortex lightly or mix by flicking the tube
   Pulse down

5. Cover the reaction tube with tin foil and store in a drawer at room temperature (23°C) for 1-2 hrs

Purification of the First Strand

1. Add 700 µL of Binding buffer (with Isopropanol added) to a new 1.5 mL tube

2. Add neutralized cDNA to Binding buffer
   Mix by flicking tube or inversion

3. Add binding buffer/ cDNA solution to a Low-Elution volume spin column
   Spin at 3,300 x g for 1 min at 23°C
   Discard the flow-through

4. Add 600 µL of wash buffer to the spin column
   Spin the column at 10,000 x g for 30s at 23°C
   Discard the flow-though

   Spin the column again at 10,000 x g for 30s at 23°C
   Place the column in a new amber collection tube

5. A 15 µL of DEPC-treated H_2O to the centre of the spin column
   Incubate at room temperature (23°C) for 1 min

Spin at 10,000 x g for 1 min at 23°C
Keep eluate

Add an additional 15 µL of DEPC-treated H_2O to the spin column
Incubate at room temperature (23°C) for 1 min
Spin at 10,000 x g for 1 min at 23°C

6. Store the cDNA at -20°C for up to 1 week
At -80°C for more than 1 week
At 4°C for immediate use

Appendix I – aRNA labelling SOP

Created by Fraser Clark (AVCLSC). 2010.

Reagent Kits Required:

Invitrogen SuperScript™ Plus Indirect cDNA Labelling System	Cat # L1014-06
Ambion MEGAclear™ Kit	Cat # AM1908M
Ambion cDNA Filter Cartridges and Tubes	Cat# AM10066G

Labelling of aRNA with AlexaFluor Dyes

1. Dry aRNA to 0-0.5 µL in speed vac

   Note: Be careful not to dry the pellet too much or it will be hard to resuspend the aRNA

2. Add 9 µL of coupling buffer to the dried aRNA

3. Add 11 µL of DMSO to a vial of AlexaFluor Reactive Dye
   Vortex to resuspend the dye
   Pulse briefly

4. Add the DMSO/dye solution to the aRNA/coupling buffer solution
   Mix by flicking the tube
   Pulse briefly

5. Incubate the tube at room temperature (23°C) in the dark for 1-2 hrs

Purify the labelled aRNA

1. Add 105 µL of aRNA binding solution (MEGA Clear Kit) to the labelling reaction

2. Add 75 µL of 100% EtOH
   Mix gently by flicking or inversion

3. Add solution to a cDNA low elution column
   Spin at 10,000 x g for 1 min at 23°C
   Discard the flow-through

4. Add 500 µL of wash buffer to the column
   Spin at 10,000 x g for 1 min at 23°C
   Discard flow-through

Spin at 10,000 x g for 1 min at 23°C
Discard flow-through
Place the column in a new collection tube

5. Add 10 µL of nuclease-free water (preheated to 55°C)
   Incubate the column/collection tube in the heating block at 55°C for 2 min
   Spin at 10,000 x g for 1.5 min at 23°C

6. Add an additional 10 µL of nuclease-free water (preheated to 55°C)
   Incubate the column/collection tube in the heating block at 55°C for 2 min
   Spin at 10,000 x g for 1.5 min at 23°C

7. Store aRNA at 4°C if you intend to use it that day (you should),
   -20°C if you intend to use the next day
   -80°C to store for several days

NanoDrop your results

Appendix J – aRNA Fragmentation SOP

Created by Fraser Clark (AVCLSC). 2010.

Reagent Kits Required:

Ambion 10X Fragmentation Reagent Cat # AM8740

aRNA Fragmentation (2-20ug)

1. Bring aRNA sample volume to 9 µL with nuclease-free H2O
2. Add 1 µL of 10X Fragmentation buffer to aRNA
3. Mix, pulse briefly and incubate at 70^{o}C for 10 min

   Use a thermocycler with heated lid
4. Add 1 µL of Stop Solution and store on ice until ready to use

   Don't forget to multiply your starting aRNA concentration by 9/11 to determine your final concentration of aRNA.

Appendix K – Microarray Hybridization SOP

Created by Fraser Clark (AVCLSC). 2010.

For use with Tecan 400 HS Pro and A2 hybridization chambers

Hybridization Mix Preparation

1. Thaw hybridization mix, ensure all contents are thoroughly mixed

2. Add appropriate reagents for 62.5 µL of hybridization mixture

Component	Volume (µL)
Labelled cDNA and H2O	11.5
Ambion Hyb Buffer #3	50
LNA dT blocker (Genisphere Cat # CW3910)	1

3. Incubate hybridization mixture at 80°C for 10 min and then hold at 65°C until sample injection

4. Inject the entire volume of hybridization mixture into the hybridization port on chamber

Tecan 400 HS Pro Preparation

1. Turn on computer and log in to HS Pro manager

2. Turn on hybridization station
   Switches are at the back and front of hybridization station

3. Place hybridization buffers on hybridization station and add the appropriate tubes to the buffers

4. Turn on N_2 tank
   Pressure gauges on the tank regulator should read 39 kPa and >100 psi

5. Carefully install hybridization chambers and microarray slides
   Ensure dummy slides are added if less than 4 slides are used

6. Open the desired hybridization program

7. Purge the system (channel 1)

8. Start the program

9. Ensure that the system program is set up properly
   i.e. Desired chambers (A2) and the number of arrays are properly selected

10. Add hybridization mix when prompted by the hybridization station

11. Press the arrow on the hybridization station after each sample is added

Tecan 400 HS Pro Clean-up

1. Remove arrays once prompted by the hybridization station

   Scan arrays using the GenePix 4000B scanner

2. Rinse the hybridization station with diH_2O and run final dry

3. Logout of HS Pro manager

   Turn off hybridization station

   Turn off N_2 tank

Liquid Definition

Channel 1: 5X SSC, 0.01 % SDS, 0.2% BSA
Channel 2: 2X SSC, 0.2% SDS
Channel 3: 0.2X SSC
Channel 4: 5X SSC
Channel 5: 0,2X SSC, 0.2% SDS
Channel 6: diH_2O

Hybridization Protocol

1.Hybridization: Temp. °C: 65, Agitation Frequency: No, High Viscosity Mode: No, Time: 0:10:00
2. Wash: Temp. °C: 65, First: Yes, Ch: 1, Runs: 1, Wash Time: 0:00:20, Soak Time: 0:00:00
3. Wash: Temp. °C: 50, First: No, Ch: 1, Runs: 1, Wash Time: 0:00:20, Soak Time: 0:00:00
4. Hybridization: Temp. °C: 50, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 0:13:00
5. Wash: Temp. °C: 50, First: No, Ch: 1, Runs: 1, Wash Time: 0:00:20, Soak Time: 0:00:00
6. Hybridization: Temp. °C: 48, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 0:13:00
7. Wash: Temp. °C: 45, First: No, Ch: 1, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
8. Wash: Temp. °C: 48, First: No, Ch: 4, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
9. Sample Injection: Temp. °C: 48, Agitation: Yes, BCR: No
10. Hybridization: Temp. °C: 48, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 16:00:00
11. Wash: Temp. °C: 40, First: No, Ch: 2, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
12. Hybridization: Temp. °C: 40, Agitation Frequency: Medium, High Viscosity Mode: No, Time: 0:02:00
13. Wash: Temp. °C: 30, First: No, Ch: 2, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
14. Wash: Temp. °C: 30, First: No, Ch: 5, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
15. Hybridization: Temp. °C: 30, Agitation Frequency: Medium, High Viscosity Mode: No, Time: 0:02:00
16. Wash: Temp. °C: 23, First: No, Ch: 5, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
17. Wash: Temp. °C: 23, First: No, Ch: 3, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
18. Hybridization: Temp. °C: 23, Agitation Frequency: Medium, High Viscosity Mode: No, Time: 0:02:00
19. Wash: Temp. °C: 23, First: No, Ch: 3, Runs: 1, Wash Time: 0:01:00, Soak Time: 0:00:00
20. Slide Drying: Temp. °C: 30, Time 0:03:00, Final Manifold Cleaning: Yes, Ch.: 6

Appendix L – SpotReader parameters

Flag features that have:	
Saturated Foreground	>50% of pixels are saturated in either channel
Uneven Colour	0.75 is greater than colour purity of foreground pixels
Bright Specks	4 is less than the 90^{th}-percentile/median
Low Foreground	1.2 is greater than foreground/background
High Background	10 is less than brightest background sector/average background sector
Low Background	0.1 is greater than lowest background sector/average background sector
Variable Background	5 is less than highest background sector/lowest background sector
Offset Centers	Greater than 0.5 times the average vertical feature spacing
Small Diameter	Less than 4 pixels in diameter
Relatively Small Diameter	Less than 0.5 times average diameter in block
Relatively Large Diameter	Greater than 1.5 times average diameter in block

Appendix M – PCR Primer Design SOP

Modified from Fraser Clark (AVCLSC). 2011.

1. Obtain desired template sequence from public repositories (GenBank, NCBI databases, research articles, etc.)

2. Determine the location, if any, of secondary structures, namely hairpin loops. This can be done using software such as the DINAmelt server for two-state melting.

Energy Rules	
Nucleic Acid	DNA
Temperature	60°C
$[NA^+]$	50
$[Mg^{++}]$	5
Measure type	mM
Sequence type	Linear

3. With location of secondary structures identified, design primers using online primer design software, such as Primer3Plus.

Criteria	
Design of Primers	Left(Forward) and (Right) Reverse
Sequence ID	Identification for the job
Excluded Region	Enter in excluded regions, such as hairpin loops
Product Size Range	If a desired product size is require
Number of Returns	Generally 25 is sufficient
Primer Size	Min:18 Max:30, usually sufficient
Primer Tm	Min: 58 Opt: 60 Max: 62
Primer GC %	Min: 40 Max: 60
[Monovalent cations]	50 mM
[Divalent cations]	50 mM

4. When picking primers 1 must be careful of the following:

Selection Criteria	
Product Size	Amplicon size should be suitable for project
Tm	Melting temperature of primer pairs within 2°C
Any	This number should be ≤ 3
Pair and Complement	This number should be ≤ 3

5. Once optimal primers are chosen they should be searched for through NCBI Basic Local Alignment Search Tool (BLAST) to ensure specificity of primers to the selected sequence. This can be done using Primer-BLAST and entering in the forward and reverse primers.

6. Once optimal and specific primers are chosen, they must be ordered through a reputable oligonucleotide synthesizing company, such as Sigma Genosys or Integrated DNA Technologies. When ordering, it is wise to order two forward and two reverse primers for each sequence to ensure the highest possible success rate of obtaining a working primer.

Markham, N.R., Zuker, M. 2005. DINAMelt web server for nucleic acid melting prediction. Nucleic Acids Research, 33: W577-W581.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M. 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Research, 35: W71-W74.

Appendix N – cDNA Synthesis for RT-qPCR

Modified from Fraser Clark (AVCLSC). 2010.

Reagent kits required:

Invitrogen SuperScript® III First Strand Synthesis SuperMix Cat #18080-400

cDNA Synthesis

1. Prepare the following:
   1 µg of total RNA
   300 ng of ovary, hepatopancreas, and eye
   100 ng of haemocyte pellet
   1 µL of oligo$(dT)_{20}$ Primer (50 µM)
   1 µL of annealing buffer
   DEPC-treated H_2O to 8 µL

2. Mix, pulse, and incubate at 6°C for 5 min at 23°C
   Use the thermocycler with a heated lid
   Place reaction on ice for at least 1 min

3. Add the following to each tube on ice:
   10 µL of 2X first strand reaction mix
   2 µL Superscript III/RNaseOUT enzyme mix
   Mix solution and pulse briefly

   Make up a No enzyme mix control (2 µL of nuclease-free H_2O in place of Superscript III/RNaseOUT enzyme mix)

4. Incubate solution at 50°C for 50 min with a heated lid
5.
6. Terminate the reactions by increasing the temperature to 85°C for 5 min
   Chill on ice

7. Add 40 µL of nuclease-free H_2O
   Mix to resuspend completely

8. Aliquot 2 µL to 30 x 0.5 mL tubes

9. Store at -80°C

10.

Appendix O – Optimization of PCR/qPCR Primers

Fraser Clark (AVCLSC). 2010.

qPCR reagents Kits Required:

Invitrogen express SYBR® GreenER™ qPCR Supermix Cat # 1179401K

Note: Primers are shipped lyophilized. Prior to first use, spin at 5000 x g for 2 minutes at room temperature (23°C). Dilute each primer to 1:10 µmol.

Reaction mix

1. Prepare the following 20 µL reaction
   10 µL 2X Express SYBR® GreenER™ Supermix
   0.4 µL Forward Primer (10 µM)
   0.4 µL Reverse Primer (10 µM)
   2 µL Template cDNA
   7.2 µL Nuclease-free H_2O

2. Cycling protocol
   50°C for 2 min (UDG incubation)
   95°C for 2 min
   40 cycles of:
      95°C for 7 sec
      Annealing temperature for 20 sec
      72°C for 20 sec
   Melt curve analysis
      65-90°C, read every 0.5°C (2 sec at each temperature)
   Use thermocycler with a heated lid

Primer Annealing Optimization – Gradient PCR

Determine the optimal annealing temperature for your primers

1. Set up a qPCR reaction

2. Run a gradient reaction with annealing temperatures from 55-70°C

The optimal temperature will give you the lowest cycle threshold (Ct) at the highest temperature with the most specificity. Melt curves will help determine specificity. A single peak generally above 80°C will be most specific.

Primer Efficiency Calculations

Determine the efficiency of the qPCR reaction for the primer set. You want between 90% and 105%.

1. Generate a serial (5 or 10 fold) dilution of cDNA

2. Run a qPCR reaction at the optimal annealing temperature
   Run dilutions in triplicate
   Run at least 3 dilutions

3. Generate a graph of log copy number (dilution) vs Ct.

4. Use an online calculator to calculate the efficiency from the slope
   -3.322 is and efficiency of 100%

Appendix P – Agarose Gel SOP

Fraser Clark (AVCLSC). 2010.

1. Weigh out 0.45 g of agarose on weigh paper.

2. Add the agarose to a clean Erlenmeyer flask.

3. Add 30 mL of 1X TBE (Tris/Borate/EDTA) to the flask.

4. Partially cover the flask with plastic wrap and the microwave. Microwave 30 mL for 2x 30s ensuring not to boil.

5. Remove the flask from the microwave after all of the agarose is in solution. Add 3-4 µL of SYBR Safe and mix well.

6. Clean the gel holder with EtOH and then with distilled water. Dry the holder with Kimwipes and place into the horizontal electrophoresis apparatus.

7. Clean the well casting combs with EtOH and then distilled water. Dry the combs with Kimwipes and place into the gel holder.

8. Insert the gel holder perpendicular to the apparatus and insert the casting combs.

9. Once you can hold the Erlenmeyer flask, add the agarose gel solution to the gel holder. Pour the gel into one corner slowly so that no bubbles are introduced.

10. Allow the gel to harden.

11. Carefully remove the casting combs and align the gel parallel inside the apparatus.

12. Cover the gel with 1X TBE.

13. Prepare your samples by adding 2 µ L of 6X loading buffer to 10 µ L of sample.

14. Load sample to the wells. Make sure to leave at least 1 empty/row for your molecular weight marker (6 µL).

15. Place the cover on the electrophoresis apparatus and run the gel at 90 V until the dye front has migrated through 2/3 of the gel.

> Note: The DNA will migrate toward the anode (red) so make sure that you have your gel properly positioned

16. Retain the overlay buffer by pouring it back into the overlay buffer bottle.

17. Lightly rinse the gel using distilled H_2O

18. Scan using the VersaDoc 2D Imager

10X TBE Recipe

Tris Base	108 g
Boric Acid	55 g
EDTA	7.44 g
diH_2O	to 1 L

Appendix Q – qPCR set up and run

Fraser Clark (AVCLSC). 2011.

qPCR reagents Kits Required:

Invitrogen express SYBR® GreenER™ qPCR Supermix Cat # 1179401K

Reaction mix

1. Prepare the following 1.2 mL reaction
   665 µL 2X Express SYBR® GreenER™ Supermix
   25 µL Forward Primer (10 µM)
   25 µL Reverse Primer (10 µM)
   485 µL Nuclease-free H_2O

2. Remove Template cDNA from -80°C freezer
   Dilute to 40 µL with nuclease-free H_2O

3. Insert 96-well plate into the QIAgility™ machine. Set up proper protocol and ensure tips are filled properly.

4. Once the plates are filled, place 1 plate in the Chromo4 and cycle as followed:

   Cycling protocol
   50°C for 2 min (UDG incubation)
   95°C for 2 min
   40 cycles of:
   95°C for 7 sec
   Annealing temperature for 20 sec
   72°C for 20 sec
   Melt curve analysis
   65-90°C, read every 0.5°C (2 sec at each temperature)
   Use thermocycler with a heated lid

Appendix R – Lobster Ovary Stages

Ovary stages for all female lobsters collected. Lobster IDs were unique names given to each biological sample. Ovary stages were determined using a combination of techniques including ovary colour, oocyte size, and ovary factor. Lobsters classified as "Unstaged" were unable to be classified as 4a or 4b using current techniques. Lobsters with 1 asterisk (*) were used in microarray analysis only; lobsters with two asterisks (**) were used in both microarray and RT-qPCR analyses. Lobsters with no asterisk were not included due to insufficient RNA.

Lobster ID	Ovary Stage
F1	3
F2	2
F3 *	3
F4	3
F5 *	3
F6 *	4b
F7 *	3
F8	2
F9 *	3
F10	3
F11 *	4a
F12	2
F13	2
F14 **	3
F15 **	Unstaged 4
F16	2
F17 **	4a
F18	4a
F19 **	4a
F20 *	4a
F21 *	4a
F22 **	Unstaged 4

Lobster ID	Ovary Stage
F23 **	4b
F24 **	5
F25 **	5
F26 **	5
F27 **	5
F28 **	5
F29 **	5
F30	5
F31 **	4a
F32 **	2
F33 **	2
F34 **	2
F35 **	2
F36 **	4a
F37 **	Unstaged 4
F38 **	4a
F39 **	2
F40 **	3
F41 **	2
F42 **	3
F43	2

Appendix S – Abbreviated List of Significantly Different Genes

The following tables (R1-R5) contain statistically significant genes determined by 1-way ANOVA. Figure of merit analysis determined 5 clusters was the optimal number for these genes. Overall expression was determined for each cluster and plotted accordingly (Figure 2.3). HAZ identification (id) numbers are unique lobster oligonucleotide probe identification codes which correspond to specific ESTs. Protein name and source organism was determined using Blast2Go software in which the best hit for each EST was determined. Unadjusted p-values were determined by 1-way ANOVA. P-values were corrected for false discovery using Benjamini-Hochberg (B-H) correction.

Table S1 – Significantly different (unadjusted p-values) genes grouped into k-means cluster 1 (n=276). The average expression profile increased to ovary stage 3 then became downregulated to stage 5. The cluster contained 52% functional annotation. Genes containing an asterisk (*) were used for RT-qPCR analysis.

HAZ Number	EST Acc Number	Protein Name	Source Organism	Unadjusted p-value	B-H Adjusted p-value
Growth/Development					
HAZ17926	FE841421	AT-rich interactive domain containing protein 5 B	Danaus plexippus	0.001145	0.033338943
HAZ04777	DV772283	Super sex isoform b	Drosophila melanogaster	0.01084	0.098750224
HAZ09100	EH401275	Inhibitor of growth *	Acromyrmex echinatior	0.01311	0.108653878
HAZ00274	FD584754	Growth hormone inducible transmembrane protein *	Nasonia vitripennis	0.019365	0.131763968
HAZ00416	FD585221	Cuticle protein AM1199	Cancer pagurus	0.034403	0.175680707
Reproduction					
HAZ00736	FD483496	Farnesoic acid o-methyltransferase	Nilaparvata lugens	0.002607	0.047219746
HAZ01770	FD584577	Ovary-development related protein *	Eriocheir sinensis	0.008897	0.088011862
HAZ07892	EH035370	Lysine-specific histone demethylase 1	Crassostrea gigas	0.013202	0.108715723
HAZ17438	FE659902	Ovary-development related protein *	Eriocheir sinensis	0.016537	0.121407699
HAZ16854	FE535321	Estradiol 17-β dehydrogenase *	Saccoglossus kowalevskii	0.022984	0.142559923

Immune Function					
HAZ07607	EH116404	Cathepsin C	Fenneropenaeus chinensis	0.021367	0.13738981
Steroidogenesis					
HAZ17540	FE660113	Cytochrome C	Panulirus argus	0.008681	0.086540822
Other Significant Genes					
Unannotated Genes: 132		Annotated Genes: 132			

Table S2- Significantly different (unadjusted p-values) genes grouped into k-means cluster 2 (n=251). The average expression profile of this cluster was downregulated. The profile decreased to ovary stage 3 then increased to stage 5. The cluster contained 25% functional annotation. Genes containing an asterisk (*) were used for RT-qPCR analysis.

HAZ Number	EST Acc Number	Protein Name	Source Organism	Unadjusted p-value	B-H Adjusted p-value
Energy Regulation					
HAZ15752	FD585372	Triosephosphate isomerase	Penaeus monodon	0.001795	0.040029538
HAZ12140	EW691258	Mitochondrial ATP synthase F0 complex subunit C	Callorhinchus milii	0.002671	0.047928921
HAZ07263	EG949419	Cytochrome C oxidase subunit VIIa polypeptide 2	Xenopus laevis	0.02518	0.150494872
Immune Function					
HAZ13627	FC071702	Leukocyte receptor cluster member 4 protein	Culex quinquefasciatus	0.004304	0.062074138
HAZ07420	EW703201	Leukocyte receptor cluster member 9	Caligus rogercresseyi	0.021686	0.138632292
Fertilization/Reproduction					
HAZ10767	EX471333	Bardet-deibl syndrome 1	Strongylocentrotus purpuratus	0.032463	0.171095975
HAZ11280	EX486935	97 kDa heat shock protein *	Strongylocentrotus francisanus	0.037847	0.185061757
Growth					
HAZ14889	FE535119	d-β-hydroxybutyrate mitochondrial-like	Takifugu rubripes	0.014902	0.116380397

Stress Response					
HAZ03347	FE660144	Heat shock protein 21	Macrobrachium rosenbergii	0.01671	0.121866125
Other Significant Genes					
Unannotated Genes: 183	Annotated Genes: 53				

Table S3 - Significantly different (unadjusted p-values) genes grouped into k-means cluster 3 (n=548). The average expression profile was downregulated to ovary stage 4a then became upregulated in stages 4b and 5. The cluster contained 29% functional annotation. Genes containing an asterisk (*) were used for RT-qPCR analysis.

HAZ Number	EST Acc Number	Protein Name	Source Organism	Unadjusted p-value	B-H Adjusted p-value
Growth/Development					
HAZ90464	FC071739	Cuticle protein	Aphis gossypii	0.000259	0.020817125
HAZ13907	FD699738	Ecdysone-inducible protein 75	Blattella germanica	0.00112	0.033110805
HAZ16272	FD699912	Ecdysone-inducible protein L3	Cherax quadricarinatus	0.003512	0.055078439
HAZ15866	FD699117	Fas-associated factor 1	Locusta migratoria manilensis	0.020102	0.134408173
HAZ02294	EH116618	Sorting nexin-12	Camponotus floridanus	0.02013	0.134478857
HAZ17844	FE841270	Innexin inx2-like	Homarus gammarus	0.023729	0.145081588
Reproduction					
HAZ09211	FD699965	Egg-derived tyrosine phosphatase *	Drosophila melanogaster	0.011443	0.101254803
HAZ10408	EW997929	Vitellogenin *	Cherax quadricarinatus	0.015034	0.116702559
HAZ05730	FD773677	Ovary-development related protein	Eriocheir sinensis	0.02233	0.141228098
Immune Function					
HAZ03881	FE535180	Kazal-like serine protease inhibitor EPI9	Phytophthora infestans	0.00179	0.040150116

HAZ14778	FD483016	ETS domain-containing protein Elk-1	Crassostrea gigas	0.025503	0.151370114
Energy Regulation					
HAZ08407	EH116279	Mitochondrial NADP+ isocitrate dehydrogenase 2	Artemia franciscana	0.032739	0.171380003
Other Significant Genes					
Unannotated Genes: 350		Annotated Genes: 148			

Table S4 - Significantly different (unadjusted p-values) genes grouped into k-means cluster 4 (n=517). The average expression profile was downregulated across all stages. Expression bottomed out at stage 4a before increasing in stages 4b and 5. The cluster contained 28% functional annotation. Genes containing an asterisk (*) were used for RT-qPCR analysis.

HAZ Number	EST Acc Number	Protein Name	Source Organism	Unadjusted p-value	B-H Adjusted p-value
Growth/Development					
HAZ08352	EH116194	Quaking protein A *	Culex quinquefasciatus	0.000821	0.030165886
HAZ17973	FE841507	Lola protein isoform A	Drosophila melanogaster	0.001228	0.033961462
HAZ10243	FF277159	Innexin 3	Papilio xuthus	0.007126	0.07900031
HAZ17510	FE660056	C-terminal-binding protein	Camponotus floridanus	0.011961	0.104283702
HAZ11029	EX486516	Cuticle protein 18.6, isoform B	Lepeophtheirus salmonis	0.031338	0.168387192
HAZ18367	FF277856	3-hydroxy-3-methylglutaryl-coenzyme A reductase-like	Bombus impatiens	0.043658	0.196767014
Immunity					
HAZ04557	DV771966	Serine protease inhibitor 5	Penaeus monodon	0.001903	0.040674648
HAZ06425	DV774710	Signal peptide peptidase-like 2b-like	Metaseiulus occidentalis	0.032037	0.17001203
Reproduction					
HAZ11967	EX568231	Vitellogenin *	Homarus americanus	0.00004	0.016244211
HAZ07899	EF422415	Vitellogenin (HaVg1) *	Homarus americanus	0.000186	0.020799652

Other Significant Genes	
Unannotated genes: 406	Annotated genes: 132

Table S5 - Significantly different (unadjusted p-values) genes grouped into k-means cluster 5 (n=182). The average expression profile was upregulated across all stages. The profile remained stagnant before increasing in stages 4b and 5. This cluster contained 33% functional annotation. Genes containing an asterisk (*) were used for RT-qPCR analysis.

HAZ Number	EST Acc Number	Protein Name	Source Organism	Unadjusted p-value	B-H Adjusted p-value
Development					
HAZ02570	CN950688	Distal-less	Parhyale hawaiensis	0.039584	0.189003802
HAZ10431	FC556629	Niemann-pick type C-2a	Drosophila melanogaster	0.04573	0.201514951
Reproduction					
HAZ04163	FC071813	Dopamine β-hydroxylase	Homarus americanus	0.003211	0.052491686
HAZ14456	FD467667	Vitellogenin *	Metapenaeus ensis	0.004872	0.064926342
Other Significant Genes					
Unannotated Genes: 127		Annotated Genes: 56			